

Handbook of Cancer Vaccines

Edited by

Michael A. Morse, MD
Timothy M. Clay, PhD
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HANDBOOK OF CANCER VACCINES

CANCER DRUG DISCOVERY AND DEVELOPMENT

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PREFACE

Harnessing the immune system to destroy tumors has undeniable appeal. Current cytotoxic cancer therapies are relatively indiscriminate, with narrow therapeutic windows. Immune effectors have the capacity to precisely target cells for destruction. Cytotoxic therapies often fail because of tumor cell resistance. The immune system can adapt to ever-changing challenges. Cytotoxic therapies are viewed as poisons, whereas immune-mediated cell death is viewed as natural.

The immune system has two arms, cellular and humoral, and each immunotherapy can be broadly described by which arm mediates its activity. Immunotherapies can also be classified by whether they activate immune effectors or involve their passive transfer. For example, active immunotherapy involves immunization to activate T cell or antibody responses *in vivo*, whereas passive immunotherapy involves the infusion of T cells or antibodies activated or generated *ex vivo*. Although the most commercially successful products thus far have been monoclonal antibodies administered intravenously, animal models have suggested that immunizations can be used to protect against tumor challenge and more importantly, to destroy established tumors. Over the last two decades, the identification of antigens that can serve as targets for immune effectors and the elucidation of the mechanisms for activating antigen-specific immune responses have resulted in a profusion of strategies for activating tumor antigen-specific immune responses. These so-called therapeutic vaccines, unlike prophylactic vaccines for the prevention of infections, all share some basic attributes, the presence of target antigens, and a method for delivering the antigen into the antigen-presentation machinery in conjunction with other molecules required to provide T- and/or B-cell activation.

The *Handbook of Cancer Vaccines* is intended to provide a comprehensive description of the scientific background for therapeutic vaccines, the challenges to their development, and their current use to treat cancer. After an overview of the immune response to cancer vaccines, this text will describe methods of antigen discovery followed by individual chapters on basic issues regarding all vaccines, such as immune adjuvants and prime-boost strategies. Subsequently, chapters will be devoted to the scientific basis and pre-clinical development of the major vaccine strategies, such as peptide, tumor cell, and dendritic cell vaccines. The last half of the text describes the clinical results for cancer vaccines used to treat many of the common cancers. Finally, chapters are devoted to the monitoring of biologic responses to vaccines and to statistical and regulatory issues affecting the design and conduct of clinical trials of cancer vaccines.

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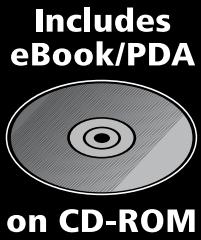
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I

BASIC SCIENCE OF TUMOR ANTIGENS AND IMMUNE ACTIVATION

1

Biology of the Cancer Vaccine Immune Response

*Michael A. Morse, MD, Timothy M. Clay, PhD,
and H. Kim Lyerly, MD*

CONTENTS

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 - HOW CELLULAR EFFECTORS RECOGNIZE TUMORS
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1. INTRODUCTION

Interest in activating the immune system to control tumors dates to more than 100 yr ago and is often attributed to William B. Coley who used the inflammatory response to bacterial products as a form of immunotherapy (1). This initiated the era of attempts to nonspecifically activate the immune system with the hope that tumors would be targeted as part of the overall, predominantly inflammatory, response. Following the abandonment of the crude bacterial products in Coley's toxin, other bacteria or their cell wall products such as bacille Calmette-Guérin (BCG), *Corynebacterium parvum*, nocardia rubra, OK-432, and others have been tested. In some circumstances, these inflammatory activators have had success as antitumor agents. For example, BCG has activity in superficial bladder cancer *in situ* (2). Later, cytokines such as interleukin (IL)-2 that could activate T and natural killer (NK) cells were extensively tested and demonstrated activity in renal cell carcinoma and melanoma. In the past two decades, a new era in cancer immunotherapy has opened with the discovery of how the immune system can recognize and destroy tumors. This has included the description of tumor-expressed antigens that can be recognized by immune effectors and a characterization of how immune effectors are primed to recognize these antigens as a signal to destroy the associated tumor. By

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harnessing these observations, it has been possible to design vaccines with the necessary ingredients for activating tumor antigen-specific immune responses *in vivo*.

2. HOW CELLULAR EFFECTORS RECOGNIZE TUMORS

It has long been realized that many tumors are poorly immunogenic. That is, if they are merely disaggregated and reinjected, they frequently grow unabated and do not activate a protective immune response. Subsequently, it was demonstrated that tumors could be modified to increase their immunogenicity. After immunization with these modified tumors, the immune system could recognize and destroy the original unmodified tumor (3). These data demonstrated that tumors could be recognized by T cells once the T cells had been activated.

It is now known that activation of naïve, tumor antigen-specific cytotoxic T cells (CTLs) so that the T cell proliferates and becomes capable of tumor destruction requires at least two signals. The first signal is the presentation of tumor antigen in the form of an 8–10 amino acid epitope within the major histocompatibility complex (MHC) class I (in humans, called human leukocyte antigens [HLA]A, B, C) molecule to the T-cell receptor (TCR). The second signal is engagement of the T-cell CD28 molecule with costimulatory molecules (CD80 and CD86) on the stimulating cell. Without this costimulation, antigen-specific T cells enter a state of anergy. Although tumors frequently express MHC molecules containing tumor peptides, they do not possess the necessary costimulatory molecules (4). *In vivo*, these two signals are provided by antigen-presenting cells such as dendritic cells (DCs). Therefore, strategies for activating immune responses against tumors involve either delivering antigen to antigen-presenting cells (APCs) or modifying tumors so that they can provide both signals for T-cell activation.

3. COMPONENTS OF THE IMMUNE SYSTEM INVOLVED IN TUMOR DESTRUCTION

A model of antitumor immunity that only includes cytolytic T cells is of course too simplistic. First, the activity of CTL is modified by positive influences from CD4+ helper T cells and negative influences from regulatory and suppressor T cells. Second, non-MHC-restricted tumor killing by NK cells may also be important for tumors that escape recognition by downregulation of MHC. Third, the other arm of the immune system, humoral or antibody-mediated immunity, may play an important role. Indeed, in some vaccine strategies, outcome correlates most with antibody titer. Fourth, cytokines and chemokines, elaborated by both immune effectors and tumors, may dramatically modify the activity of cellular components of the immune response.

3.1. Cytotoxic T Lymphocytes and NK Cells

Cytotoxic CD8+ T cells recognize MHC-restricted peptides on the tumor surface via their T-cell receptor. In contrast, mature NK cells express an array of germline-encoded activating and inhibitory receptors on their surface and it is the balance between these receptors (5) that determines the cytolytic activity of the NK cell. In particular, the inhibitory receptors such as the human killer cell Ig-like receptors (KIRs) recognize HLA-A, -B, and -C with bound peptide and suppress NK-cell lysis of target cells that express these class I MHC molecules, but allow the lysis of class I negative cells. Thus,

tumors that downregulate MHC become less susceptible to attack by CTLs, but more susceptible to attack by NK cells.

CTLs and NK cells share two different mechanisms for tumor-cell killing: the Fas-Fas-ligand (FasL) pathway (6), and the perforin-granzyme pathway (7). FasL, a member of the TNF (tumor necrosis factor) family, is expressed predominantly on activated T cells, macrophages, and neutrophils. Binding of a FasL trimer on the effector cell surface to a Fas trimer on the target cell membrane causes the formation of the death-inducing signaling complex (DISC) around the cytoplasmic chain of Fas. This results in interaction of Fas with the adaptor protein Fas-associated death-domain protein (FADD) and the initiation of a chain of events eventuating in apoptosis of the tumor cell target. In the perforin–granzyme pathway, perforin, a pore-forming molecule stored in cytotoxic granules together with granzymes, is released with the granules upon recognition of a target cell. Perforin monomers insert themselves into the target cell membrane and create pores that cause osmotic lysis of the target cell and allow granzymes to enter the target cell and induce apoptosis through various downstream effector pathways.

3.2. T-Helper Cells

CD4+ T-helper (Th) cells promote the activity of other immune cells, by both cytokine release and direct cell–cell interactions. Their TCRs recognize antigen in the form of peptides presented by MHC class II molecules. Since tumors generally do not express MHC II, Th cells are primed by APCs such as DCs or B cells. Different subsets of Th cells, designated Th1 and Th2, have pronounced differences in their effect on the immune response. The Th1 response, characterized by secretion of IL- and interferon- γ (IFN- γ), is stimulatory for CTLs, whereas the Th2 response, characterized by secretion of IL-4, IL-5, IL-6, and IL-10, is important for isotype switching and antibody production by B cells. Activated Th cells also provide direct signals for maturation of DCs, via expression of CD40-ligand, which binds to CD40 on DCs. CD40L is also an important signal for B-cell isotype switching. Whether a Th cell follows the Th1 or Th2 pathway is dependent on signals from APCs such as IL-12, which skews the response toward Th1. Because numerous studies have demonstrated that the induction of Th1 responses can slow or prevent tumor growth, whereas Th2 responses may permit tumor growth, it is generally thought that anticancer vaccines should contain adjuvants that induce Th1-type cytokines. There is also increasing interest in designing vaccines that contain MHC class II epitopes in addition to MHC class I epitopes to ensure the participation of Th cells in activating the immune response (8).

3.3. B Cells

B cells recognize tumor antigen as epitopes on proteins or carbohydrates via the B-cell receptor, a surface-expressed, monospecific immunoglobulin. Although B cells can be directly activated by large antigens that bind simultaneously to multiple antibody receptors, this T-cell-independent activation results in mainly IgM production and poor memory induction. T-cell-dependent responses, in contrast, usually require two signals for activation, antigen binding to the B-cell receptor and cytokine secretion from Th cells. Antigen either binds to the B-cell receptor or is presented to B cells by APCs such as macrophages or follicular DCs. Antigen is also processed by APCs and presented to Th cells. Furthermore, antigen taken up by B cells can be processed and presented to Th cells via MHC class II molecules. The Th cells supply IL-2, IL-4, IL-5, and CD40-ligand that

lead to B-cell proliferation, class switching, and the B cell eventually becomes an antibody-secreting plasma cell or a memory B cell. Although the natural antibody response against tumor cells is weak, monoclonal antibodies are now being used to treat lymphomas and breast cancer successfully. Furthermore, some of the most promising tumor vaccines in later stage clinical trials activate antibody responses. There is now increasing emphasis on attempting to include humoral immunity in anticancer vaccine approaches (9). Similarly, immunologic monitoring of vaccine clinical trials is assuming increasing importance.

Antibody-mediated killing of tumors may occur by antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). ADCC occurs when the Fc region of an antibody interacts with Fc receptors (FcRs), FcRI and FcRIII, on immune effector cells such as macrophages, NK cells, and neutrophils. The effector cell then destroys the tumor by phagocytosis or lysis. In CDC, recruitment of the complement component C1q by IgG bound to the tumor-cell surface triggers a proteolytic cascade that results in cell death in one of two ways. First, the proteolytic cascade may ultimately result in formation of a membrane attack complex that kills the target cell by rupturing its cell membrane. Second, tumor-cell-bound C1q can bind to complement receptors, such as C1qR, CR1 (CD35), and CR3 (CD11b/CD18), on macrophages, NK cells, and neutrophils, triggering phagocytosis or lysis.

3.4. Dendritic Cells

Because of their central role in activating adaptive immune responses, dendritic cells (DCs) have garnered the most recent attention in the development of anticancer vaccines. Derived from bone marrow precursors, DCs circulate to peripheral sites (such as the epidermis of the skin where they are called Langerhans cells), capture and process antigen, and traffic to draining lymph nodes where they prime CTL, Th, and B cells. Exogenous antigens delivered in vaccines can enter both the class II and class I MHC pathways within DCs resulting in their ability to activate both CD4+ Ths and CD8+ CTLs. The potency of DCs as T-cell stimulators derives from several factors, the high level of expression of MHC, costimulatory, and adhesion molecules, that permit the formation of strong “immuno-logic synapses” (10) at the site of T-cell–DC interactions. Because only immature DCs efficiently take up and process antigen, whereas mature DCs are the most effective at antigen presentation, the most successful vaccine strategies may be those that interact with DCs to achieve the desired maturation state. Furthermore, DCs can be divided into DC1s and DC2s. DC1s are immunostimulatory, but DC2s are frequently described to be tolerogenic. Therefore, vaccine strategies that can promote DC1 effects over DC2, may be important. Finally, the ability to generate DCs ex vivo has also spawned a large number of studies using antigen-loaded DCs as the cancer vaccine.

3.5. NKT Cells

Natural killer T cells (NKTs), a subset of lymphocytes that express both T-cell and NK-cell markers, recognize glycolipid antigen (GalCer) presented by the MHC-like molecule CD1d. Although few in number, they can produce large amounts of IL-4 or IFN- γ , and promote humoral or cellular immunity. In common with NK cells and CTLs, they kill tumor cells by a perforin-dependent mechanism. NKT are thought to play a role in biasing the immune response toward TH1 or TH2, and have been implicated in immune

surveillance and protection against carcinogenesis (11). A regulatory or immunosuppressive subset of NKT cells has also recently been reported. It is still uncertain how best to integrate an understanding of these cells into vaccine approaches, but the availability of the NKT ligand alpha-galactosylceramide (KRN7000) has permitted further study.

3.6. Regulatory T Cells

As is the case with all biologic systems, the immune response is characterized by the need for regulation to prevent excessive or aberrant immune responses from causing auto-immunity. Several cell types mediate regulation of immune responses. First, CD8+ suppressor T cells, stimulated by DC2s (12), release IL-10 and suppress the reactivity of other T cells. Another recently described regulatory T-cell subset, the CD4+/CD25+ T cells, can mediate suppression by cell–cell contact and this suppressive effect is dependent upon surface-bound transforming growth factor- β (TGF- β) on the regulatory T cells (13). Finally, some signals delivered from APCs to T cells may actually downregulate the immune response. For example, CD80 binding to CTLA-4 on T cells causes downregulation of T-cell activity. Although vaccines may in fact activate potent immune responses, it will likely be necessary to interfere with these regulatory and suppressor influences. Vaccine studies are now beginning to integrate addition manipulations to abrogate regulatory responses including the use of antibodies to deplete CD4+/CD25+ T cells or interfere with CTLA-4 activation.

3.7. Cytokines

Cytokines are critical for signaling between immune effectors. In vivo, cytokines released at the site of tissue damage from tumors, such as tumor necrosis factor- α (TNF- α), CD40 ligand (CD40L), and IFN- γ , cause DC maturation. Granulocyte macrophage-colony-stimulating factor (GM-CSF) released from macrophages, and T and B cells, also is involved in DC differentiation. DCs produce cytokines such as IL-12, IL-15, and IL-18, which drive Th1 responses. In turn, the Th1 cytokines IFN- γ , IL-2, and TNF- α appear to be important for the generation of antitumor, CTL activity as described above.

Because of the importance of cytokines to in vivo immune responses, they have been added to vaccine strategies as adjuvants or to augment the immune response following immunization. GM-CSF has been used as an adjuvant for cancer vaccines because of its ability to activate DCs. IL-2 has been administered following immunization because it induces T-cell proliferation and cytokine production, and augments TCR-mediated and TCR-independent cytolysis. IL-12 (14) has gained increasing recognition as a possible adjunct to active immunotherapy because of its pleiotropic effects including stimulation of Th1 cells, NK-cell proliferation, and augmentation of B-cell IgG production. IFN- α has been shown to enhance humoral immunity and promote isotype switching, in part through its effects on DCs. IFN- γ can augment the immunogenicity of tumor cells through the upregulation of MHC class I expression, which may be important for tumors with downregulated MHC expression.

4. DEVELOPMENT OF CANCER VACCINES

4.1. Differences Between Tumor and Infectious Disease Vaccines

There are a number of conceptual differences between existing, clinically effective vaccines approved for use in humans and those that are being developed and tested in the

setting of cancer immunotherapy. In general, vaccines approved for clinical use in the United States are applied as prophylaxis against infectious pathogens, such as hepatitis B virus and pneumococcus. Generally, these vaccines must be administered prior to the onset of an infection (as primary prophylaxis) in order to be effective, although there are rare exceptions such as the treatment of rabies (in which both specific immunization and immune globulin are administered to achieve postexposure prophylaxis). In contrast, cancer immunotherapy vaccines presently are aimed at the eradication of either a microscopic or, more commonly, macroscopic burden of tumor cells.

A second major difference is that antigens targeted by existing vaccines are those of viruses or other pathogens, and hence are highly immunogenic, foreign antigens. No vaccines currently in clinical use target human antigenic epitopes, whether they comprise mutated or nonmutated antigens. That clinically significant immune responses can even be induced by vaccination against tumor-associated antigens (TAAs) is based on evidence derived primarily from animal models rather than from human trials. Therefore, the specific methodology for effectively immunizing humans against a self- or mutated self-antigen is unknown.

Whereas most existing vaccines approved for clinical use are believed to act through the generation of protective humoral immunity, most experts believe that this will not be the case for cancer vaccines. Rather, the induction of tumor-specific cellular immunity will likely be necessary in order to induce a clinically effective immune response. Though the leading hypothesis is that a TAA-specific cytotoxic T-cell response is a critical component of this process, no vaccines approved for routine use in patients are known to exert their clinical benefit primarily through this mechanism. Ironically, although there are no clinically proven antitumor vaccines currently in standard use, two effective monoclonal antibody preparations are currently available for the treatment of malignant disease (trastuzumab and rituximab), challenging dogma that induction of successful antitumor immunity in humans depends primarily on a cellular rather than humoral host response.

4.2. Necessary Ingredients for a Successful Cancer Vaccine

Although cancer vaccines differ from vaccines against infectious organisms in their therapeutic as opposed to prophylactic use, they share some of the same requirements for success. First, there must be an antigen expressed by the tumor and immune effectors that can recognize it as their signal to initiate the cascade of events that lead to tumor destruction. Second, vaccines must present the antigen in a manner that leads to immune activation instead of anergy. Third, they must provide or recruit other sources of cytokines to promote a more vigorous immune response. The major difference between the two types of vaccine approaches is that for tumor vaccines, the target antigens are usually self antigens, not foreign ones, and the immune system is generally predisposed to anergy against self-antigens. Therefore, it has been more difficult to merely provide tumor antigens to the immune system and induce a potent antitumor response. The following sections describe how these requirements may be met for tumor vaccines.

4.2.1. TUMOR ANTIGENS FOR CANCER VACCINES

As described above, although most tumors appear to be nonimmunogenic, they in fact express antigens, either native, surface-expressed proteins or carbohydrates, or processed peptides bound within the groove of MHC molecules, that can be recognized by

the immune system. Although some antigens are unique to their tumors such as idiotype produced by malignant B cells or viral antigens from virally induced malignancies (Chapter 5), most are “self” antigens either overexpressed or mutated in the tumor. Theoretically, tumor antigens may be derived from any cellular protein. There is no consensus on whether only identified antigens or undefined pools of antigens would be preferred for a cancer vaccine. Vaccines with pools of undefined antigens (such as tumor-cell vaccines) are attractive because they permit immunization against a wider array of antigens increasing the chances that one or more of the antigens will represent a true rejection antigen in the recipient. Nonetheless, they are difficult to standardize and may face more difficult regulatory hurdles during later phases of development. Vaccines that use one or a small number of pure, defined antigens (such as peptide epitopes) are easier to produce and qualify. Immunologic monitoring is also simplified. Therefore, a major effort is ongoing to discover more antigens. Although they were previously identified by testing cDNA libraries of tumors for those that encoded antigens that could be recognized by T cells cloned from patients with malignancies, newer methods of screening for antigens have now been developed (*see* Chapters 2 and 3).

4.2.2. DELIVERY OF TUMOR ANTIGENS IN CANCER VACCINES

Merely delivering an antigen by itself is unlikely to activate a potent T-cell-mediated immune response. The antigen must be delivered in such a way that it is presented along with the necessary costimulatory molecules. This has been accomplished by mixing the antigen with an inflammatory adjuvant (Chapter 8), a carrier protein, or a cytokine such as GM-CSF to increase the exposure of the antigen to antigen-presenting cells (APCs). Also, plasmids or viral vectors containing genes for the antigen and costimulatory molecules may be administered so that when they are taken up by APCs, they can modify the APCs to express the antigen and higher levels of the costimulatory molecules. Injecting genes for costimulatory molecules directly into tumors or modifying tumor cells to express these genes and reinjecting the tumor as a vaccine can also increase the exposure of the antigen to the immune system. Finally, directly loading DCs with tumor antigens increases the likelihood that the antigen of interest will be processed and presented. The diverse array of strategies that accomplish these goals are described in Chapters 10 through 24. Combinations of strategies, most notably by prime-boost immunizations (Chapter 9), may induce the greatest immune responses. Although adoptive immunotherapy is not a vaccine strategy, the cells delivered are frequently stimulated *in vivo* or *in vitro* with vaccines and there is increasing interest in vaccinations following adoptive transfer of T cells. Therefore, Chapter 24, “T-Cell Adoptive Immunotherapy,” has been included in this text.

5. CHALLENGES TO CANCER VACCINES

Chapters 25 to 33 describe the clinical results for therapeutic vaccines in the most common malignancies. Although it is too early to determine the ultimate role for cancer vaccines, the results do provide an increasingly clear picture of the challenges that require attention. First, it will be necessary to identify from among the many strategies a few vaccines with enough promise to warrant large-scale clinical trials. This will require novel clinical trial designs and intermediate markers of activity such as immunologic assays to determine which induce the most potent antigen-specific immune responses. Recent attempts to reach a consensus on the immune assays to use and how to interpret

them (15) should simplify comparison across different studies. Clinical trial design is discussed in Chapter 34. Immunologic assays are described in Chapters 35 and 36. Second, the level of immune response detected by these assays is still fairly low. If one were to assume that the magnitude of the T-cell response necessary to clear viral infections is similar to the magnitude required to destroy tumors, then most cancer vaccines activate T-cell responses two or more orders of magnitude less than is necessary. Third, tumors possess a variety of mechanisms for evading even a high-level T-cell or antibody response. Tumor escape is addressed in Chapters 6 and 7. Finally, before a vaccine can be administered to patients, it will require considerable regulatory scrutiny to ensure that it is safe and effective. Although the regulatory requirements for infectious-disease vaccines have been honed over many years, the use of cellular vaccines poses new issues for the Food and Drug Administration and other regulators. Regulatory requirements for cell-based vaccines are discussed in Chapter 37.

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2

The Rational Design of T-Cell Epitopes With Enhanced Immunogenicity

John Fikes, PhD

CONTENTS

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1. THE CHALLENGE OF OVERCOMING IMMUNOLOGICAL TOLERANCE

For all cancer vaccine strategies, a major challenge facing efforts to induce a clinically effective T-cell response is the necessity to break tolerance to normal, “self” antigens. To control auto-reactivity, some T cells with high avidity for tumor-associated antigen (TAA) epitope-major histocompatibility (MHCs) complexes are deleted in the thymus and the remaining T cells are controlled by peripheral tolerance (1). However, several groups have demonstrated using in vitro systems that thymic-deletion of TAA-specific cytolytic T cell (CTL) is not complete (2–4). More importantly, it is clear that in some patients, natural exposure to tumor or immunization with wild-type antigens or epitopes can induce CTL of sufficient avidity and functionality to infiltrate tumors *in vivo* and/or recognize tumor cells *in vitro*. Therefore, although the fundamental vaccine strategy of targeting TAA to mount tumor-specific immune responses is supported, it remains a significant challenge to design cancer vaccine strategies that consistently overcome immunological tolerance in order to effectively activate and maintain therapeutic T-cell responses. Experimentation in the late 1980s and 1990s has resulted in a detailed understanding of the molecular mechanisms controlling T-cell activation and effector function. It is now appreciated that the interaction of a T-cell receptor with a peptide epitope presented by an antigen-presenting cell (APC) in the context of an MHC molecule generates the central event (referred to as “signal 1”) in the activation of naïve or memory

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T cells. Studies have also demonstrated that the specificity and affinity of peptide binding by human lymphocyte antigens (HLAs) is determined by the interactions between the side chains of the linear peptide epitope and the residues present in each HLA molecule. Insights from these studies have given rise to two strategies for the rational modification of T-cell epitopes to enhance immunogenicity and “breaking” of immunologic tolerance. The molecular mechanisms, immunological consequences, and the cancer vaccine applications of each approach will be discussed.

2. FIXED-ANCHOR EPITOPE ANALOGS

One type of modification that can be utilized to enhance the immunogenicity of wild-type TAA-derived peptides involves the substitution of a single amino acid residue to facilitate an increase in the binding affinity of the analog peptide for an HLA molecule. Although the affinity of peptide binding by an HLA molecule is essentially the product of the interactions between residues of the peptide and the residues in the peptide-binding cleft of the HLA molecule, two positions have a dominant influence on binding, and are referred to as “primary anchor positions.” For most HLA-binding peptides, these primary anchor residues are position 2 and the carboxyl-terminus. The specific amino acids that most effectively function as primary anchor residues in a peptide are dependent on the composition of the binding pocket of the given HLA molecule, and the amino acids that are “preferred” and “tolerated” at each peptide position have been defined for numerous HLA molecules (5). For example, for binding to HLA-A2.1, L,M and V,I,L are preferred anchors at position 2 and the C-terminus, respectively. Knowledge of these HLA-specific patterns, referred as motifs, is the foundation for the rational design of fixed-anchor analog epitopes. The primary amino acid sequence of a wild-type TAA can be analyzed using motif-based algorithms to identify peptides that harbor a preferred residue at one anchor position and a tolerated residue at the other. The HLA-binding affinity can then be enhanced by a single amino acid substitution to replace the tolerated residue, thereby “fixing” the anchor. Residues at other positions of the peptide can contribute to HLA binding, albeit more weakly, and are referred to as secondary anchor positions (5). These positions can also be substituted to enhance HLA binding of a peptide.

Unlike receptor:ligand interactions that directly result in biological functions such as signal transduction, binding of a vaccine-delivered peptide to an HLA molecule has no direct biological effect. The immunological and pharmacological importance of enhancing this interaction for vaccine design lies in the observation that the affinity of an HLA-peptide interaction directly correlates with immunogenicity (6). By designing epitopes that increase the stability of HLA-peptide complexes on the surface of APCs, the opportunity to effectively engage the cognate T-cell receptor (TCR) and induce a therapeutic T-cell response is enhanced. In addition, it has been hypothesized that CTL specific for a low-affinity, wild-type epitope may be less tolerized than those specific for high-affinity peptides, due to the lower amounts of cell-surface epitope-HLA complexes generated by normal processing and presentation (7). Therefore, by utilizing fixed-anchor analog epitopes as high-affinity immunogens, a vaccine can be used to stimulate these potentially more reactive T-cell populations.

The validity of this approach for generating immunogens with enhanced potency was initially demonstrated in *in vitro* studies using fixed-anchor analogs derived from HER-2/neu and gp100, where analogs were demonstrated to be more effective for CTL induc-

tion than the wild-type peptides from which they were derived. Single amino acid substitutions to introduce optimal residues at the anchor positions of three peptides derived from the melanoma-associated antigen gp100 were demonstrated to yield analog peptides with enhanced HLA binding and immunogenicity relative to the native sequence (8). In another early study, single amino acid substitutions were made at secondary anchor positions of HER-2/neu-derived peptides resulting in analog epitopes exhibiting enhanced HLA binding and immunogenicity (9).

Our group has utilized HLA motifs and epitope prediction algorithms in combination with high-throughput HLA binding assays to identify novel fixed-anchor analog epitopes from CEA, p53, HER2/neu, and MAGE2/3, five TAAs that are frequently expressed in epithelial-derived tumors (2,10). Initial studies focused on epitopes restricted by the HLA-A2 supertype, and included the identification of wild-type epitopes in addition to fixed-anchor epitopes. From the primary sequences of the four TAAs, approx 1650 motif-positive sequences ranging in size between 8 and 11 residues and carrying the general extended motif that has been associated with the capacity of peptides to bind HLA2.1 were identified. From these motif-positive peptides, a more refined algorithm that takes into account the influence of secondary anchors was used to select 223 peptides for further analysis. Utilizing the HLA-binding assays and this set of peptides, 82 different wild-type epitopes were demonstrated to bind HLA-A2.1 with an IC_{50} of $\leq 500\text{ nM}$, an affinity previously shown to correlate with peptide epitope immunogenicity (6). Importantly, 123 fixed-anchor analogs with optimized HLA-binding capacity were also generated. From these A2.1 binding peptides, 115 were also demonstrated to bind at least one, and most often two or three additional HLA molecules from the HLA-A2 supertype. Of these HLA-A2 supertype epitopes, 22 wild-type epitopes and 21 analogs were tested for immunogenicity in CTL induction assays using normal donor peripheral blood mononuclear cells (PBMCs). As a read-out, immunoassays measuring cytolysis and/or interferon- γ production were utilized. The CTL cultures demonstrated to be positive for recognition of the immunizing peptide were then further expanded and tested for recognition of naturally processed epitope as presented by HLA- and TAA-matched tumor cell lines. The specificity of the CTL induced by in vitro immunization with peptides was also demonstrated by cold target inhibition experiments for selected epitopes (2,10). For the wild-type peptides, 20 out of 22 (91%) were immunogenic for PBMC from at least one donor, and recognition of tumor cell lines expressing naturally processed antigens was noted for 16 out of 20 (65%) of these epitopes. Interestingly, no significant difference in “hit rate” was observed between MAGE, p53, CEA, and HER2/neu antigens, suggesting that for these TAAs the degree of peripheral tolerance in the CTL compartment is indistinguishable, despite the significantly different protein expression patterns reported for these TAAs.

Fixed-anchor analog epitopes substituted at one or both anchor positions were also evaluated for immunogenicity. Of the analog epitopes harboring a single primary anchor substitution, 100% of the analogs induced CTL when measured against target cells pulsed with the analog peptide. However, to be useful components of a cancer vaccine, epitope analogs must induce CTLs that recognize the wild-type peptide presented on the surface of tumor cells. Recognition of naturally processed antigen on tumor cell lines was demonstrated for 46% (6/13) of the fixed-anchor analogs, thereby validating their use as vaccine immunogens. Analog peptides substituted at both primary anchor positions were also found to be immunogenic, but the CTLs induced were less frequently associated with

recognition of endogenous antigens. These data underscore the importance of using rational design strategies that introduce minimal changes to the epitope, and serve as a cautionary note regarding the use of analog design approaches that yield multiply-substituted analogs.

It is interesting to consider that in general, for both wild-type and analog peptides, the induction of CTLs that are capable of recognizing tumor cell lines as targets is associated with high HLA-binding affinity (2). This conclusion is consistent with previous work by our group and others (4,8,10), although contrary to the postulate that CTL recognizing high-affinity binding epitopes are preferentially deleted from the repertoire, and that epitopes that can induce CTLs recognizing naturally processed epitopes are mostly directed against low-binding-affinity peptides (11).

Several other groups have recently identified HLA-A2.1-restricted fixed-anchor analog epitopes derived from other important TAA. Two analog epitopes derived from an overlapping region of the melanoma differentiation antigen MART-1 have been reported (12). In addition, fixed-anchor analogs derived from NY-ESO-1 (13) and Ep-CAM (14) have been identified. For each of these analog epitopes, superior immunogenicity relative to the cognate wild-type sequence was demonstrated using human PBMC in an in vitro CTL induction system.

One fixed-anchor analog tested in clinical studies is the gp100.209 (210 M) epitope, which has been utilized as a synthetic peptide delivered to melanoma patients. This analog epitope was reported to be markedly more effective at in vivo CTL induction than was the gp100.209 wild-type peptide when each was administered separately to melanoma patients as a synthetic peptide in Montanide® ISA 51 adjuvant in conjunction with IL-2 (3). In this study, the analog and wild-type peptides induced wild-type peptide-specific CTL responses in 10/11 and 2/8 patients, respectively. Although not a direct comparison of wild-type and analog peptides, two other clinical studies by Weber and colleagues (15) and Banchereau and colleagues (16) have also demonstrated the immunogenicity of the gp100.209 (210 M) analog, with the latter reporting vaccine-induced CTL recall responses in 12/16 patients. Detailed analyses of the CTL responses induced by this fixed-anchor analog epitope have been conducted (17,18).

All of the fixed-anchor epitope analogs reported to date are restricted by the HLA-A2 supertype. Although this HLA supertype is relatively frequently expressed in patients, 45% on average, vaccines produced using HLA-A2 epitopes are inherently limited in applicability. To expand the application of this rational vaccine design approach, our group is using the same strategy described above to identify CEA-, p53-, HER-2/neu-, and MAGE 2/3-derived fixed-anchor analog epitopes that are restricted by three other common HLA supertypes, -A1, -A3, and -A24 (19). The design of vaccines that combine epitopes restricted by these four HLA supertypes will provide essentially complete population coverage for all patients, regardless of ethnic background.

3. HETEROCLITIC ANALOGS

A second epitope modification strategy involves the introduction of selected single amino acid substitutions at selected positions other than the main HLA anchors of the peptide. The resulting peptides, referred to as heteroclitic analog epitopes, are capable of stimulating unexpectedly potent T-cell responses. A number of different reports have illustrated that heteroclitic analogs are associated with T-cell hyperstimulation, and that

this more potent response is, in fact, mediated by increased binding of the peptide-HLA complex to the TCR (20,21). Importantly for cancer vaccine development, heteroclitic analogs have also been associated with a striking capacity to break tolerance, as shown in a variety of different studies (22,23). Schlam and colleagues identified the first HLA-restricted heteroclitic analog epitope derived from a clinically important human TAA. This modified CEA peptide, designated CAP1-6D, harbors a single substitution of aspartic acid for asparagine at position 6 of the nine amino acid sequence (24). This substitution did not increase the HLA-A2 binding affinity of the peptide, but did result in the hyperstimulation of wild-type specific CTL when the analog peptide was presented on target cells. Importantly, under *in vitro* conditions where the wild-type peptide was not immunogenic, the CAP1-6D was demonstrated to induce human CTLs capable of recognizing HLA-A2⁺, CEA⁺ tumor cell lines. These data provided support for the use of CAP1-6D in human vaccine design (*see* later discussion), and more generally supported the concept of identification and use of heteroclitic analogs derived from human TAA.

For our initial studies to develop and characterize heteroclitic analogs (23), three wild-type, TAA-derived, HLA-A2 epitopes identified in the screening studies described earlier were selected as targets: MAGE-3.112, CEA.691, HER2/neu.157. For each epitope, conserved, semiconserved, and nonconserved substitutions were introduced at all positions in the peptide excluding the MHC anchor positions. These analogs, a total of approximately 350, were screened for heteroclicity by performing antigenicity and dose titration analyses with a CTL line specific for the cognate wild-type peptide. The magnitude of response and shifts in dose responses induced by each analog relative to the wild-type peptide were evaluated, and striking increases of the order of 6–7 logs were detected for some substituted peptides. Importantly for use of these epitopes in vaccine development, the human CTLs generated by *in vitro* immunization with heteroclitic analogs were able to recognize naturally processed wild-type epitope expressed on tumor cell lines, and were of higher avidity than CTLs induced with the parent peptide. Also, as compared to the wild-type parent peptide, heteroclitic analogs were found to more consistently induce CTLs *in vitro*. Further, a model heteroclitic analog epitope derived from the murine p53 epitope using this same analog identification strategy was demonstrated to break tolerance to “self” and activate CTLs that recognize tumor cell lines when utilized for *in vivo* immunization of HLA-A2/K^b transgenic mice.

From these studies, novel heteroclitic analog epitopes were identified, and clinical studies utilizing a subset of these heteroclitic epitopes are currently planned. In addition, several observations relating to the nature and function of epitopes displaying heteroclicity were made. First, it was noted that the amino acid substitutions generally associated with heteroclicity were conservative or semiconservative and occurred in the middle of the peptide at position 3, 5, or 7. Since odd-numbered positions in the middle of an HLA-bound peptide may be pointing up toward the T-cell receptor, this observation is consistent with the effect being mediated by an increased interaction with TCRs. Some previous studies implied that modulation of T-cell responses by heteroclitic analogs involves modification of peptide residues that directly contact the TCRs (20,24), but this finding was not corroborated by our study (23), which indicated that heteroclicity is likely to be generated by subtle alterations in epitope conformation rather than by gross alterations of TCR contacts or MHC binding capacity. Salazar et al. have reported increased phosphorylation of Zap-70 in heteroclitic analog-stimulated T cells (22), but the structural and signaling mechanisms involved in heteroclicity require further investigation.

From a practical standpoint, our studies have important implications for vaccine development. By identifying the substitution patterns most frequently associated with heteroclicity, “rules” were developed that can streamline the analog screening process. We have found that we can successfully identify heteroclitic analogs from all epitopes, and our success rate of analog prediction is in the 20–40% range, which represents about a 20-fold increase over random prediction. By combining this targeted substitution strategy with high-throughput screening, heteroclitic analog epitopes derived from any TAA and restricted by commonly expressed HLA supertypes can be identified and utilized for cancer vaccine design.

The HLA-A2 restricted, CEA-derived CAP1-6D heteroclitic analog identified by Schlam and colleagues (24) has been utilized in human clinical trials. In a study by Fong and colleagues, the CAP1-6D peptide was demonstrated to effectively expand epitope-specific human CTL when delivered using dendritic cells and measured using tetrameric HLA/peptide complexes (tetramers) (25). These CTL expansion data, and to a lesser extent the ELISPOT data reported, correlated with the observed clinical responses. Previous clinical studies using the wild-type CAP-1 peptide had demonstrated relatively weak immunogenicity (26), underscoring the potential significance of these early results obtained using the CAP1-6D heteroclitic analog. Additional clinical studies testing the CAP1-6D epitope and the novel heteroclitic epitopes described above should provide the human immunological data needed to address the general applicability of this approach for cancer vaccine design.

4. SUMMARY

The rational design of analog epitopes with enhanced immunogenicity is a promising strategy for improving on the cancer vaccine clinical outcomes to date. The use of fixed-anchor and heteroclitic analog epitopes is compatible with most all delivery systems, and these modified antigens should expand the quality and breadth of antitumor T-cell responses achievable in humans by facilitating the stimulation of CTLs specific for epitopes that in their natural form are too weakly immunogenic to be effective vaccine components. In addition, these optimized immunogens should complement other approaches directed at enhancing T-cell induction, maintenance, and effector function (1).

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3

Identification of Tumor Antigens Using Subtraction and Microarrays

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CONTENTS

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1. INTRODUCTION

Recent advances in genomic discovery approaches have led to the identification of a new generation of tumor antigens. In this chapter, we discuss the application of nucleic acid subtraction methods combined with cDNA microarrays to identify potential tumor antigen candidates. Subtraction techniques form an effective means of enriching for tissue- and tumor-specific genes whereas microarray technology provides us with an efficient high-throughput screening approach that can simultaneously determine the gene expression of thousands of genes in a single experiment. The combination of these two powerful new discovery tools allows a systematic comparison of the cancer genome with the normal tissue genome to identify differentially expressed genes. This process has been optimized for rapid, thorough, and effective gene discovery and is not dependent on the availability of immunological reagents such as antibody and T cells from cancer patients. Genes identified via this integrated approach have the advantage of being tumor- or tissue-specific and broadly expressed in cancers. Numerous tissue- and tumor-specific genes have been identified in prostate, breast, lung, and colon cancers using these approaches. Immunological validation strategies have been employed to determine the immunogenicity of these antigens and to identify naturally processed epitopes that can be used as immunogens or reagents for monitoring antigen-specific immune responses

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in subsequent vaccine strategies. With the aid of adjuvant and delivery systems, these antigens will be tested in human clinical trials and the results from these trials will undoubtedly give us insight regarding the utility of these second-generation tumor antigens.

2. FEATURES OF A TUMOR ANTIGEN

Recent advances in the identification of tumor-associated antigens and in the elucidation of mechanisms of antitumor immunity coupled with strategies designed to enhance antitumor immune responses have led to the development of more specific and potent vaccine strategies using tumor antigens. If we consider a list of features that comprise an ideal tumor antigen, it would include the following characteristics:

1. The expression of antigen is confined to tumor cells, thus allowing the vaccine to specifically target tumor cells without causing unnecessary side effects to normal tissues. Additionally, the host T-cell responses to tumor-specific antigens may be naïve, and as a result, the vaccine strategy is less likely to be limited by tolerance and anergy. Although the targeting of tumor-specific antigens presents the ideal strategy, normal tissue-specific proteins may also serve as tumor regression antigens. Targeting immune responses against tissue-specific antigens is a reasonable approach for those tissues that are not critical for survival. This has been demonstrated in melanoma, where the first three antigens identified by using human tumor-infiltrating lymphocytes were gp100, MART-1, and tyrosinase. These antigens are all normal, nonmutated proteins expressed both in melanomas and in normal melanocytes (1–4). Targeting of melanocyte-specific antigens by the immune system resulted in tumor regression with only minor side effects in patients (5).
2. The antigen is broadly expressed in tumor samples and tumor cells, permitting its use in a majority of cancer patients and allowing the targeting of a maximum number of tumor cells within malignant tissues.
3. The antigen is expressed at a sufficiently high level to allow the immune system to recognize it.
4. The antigen expression is conserved in metastatic tumor cells ensuring the elimination of distant metastases by the vaccine.
5. The antigen plays an important role in tumorigenesis allowing the vaccine to more effectively kill tumor cells and minimize the development of antigen-loss tumor variants.
6. Very importantly, the antigen needs to be immunogenic in humans to allow the induction of strong antitumor immunity.

3. IDENTIFICATION OF TUMOR ANTIGENS USING IMMUNOLOGICAL REAGENTS

Until recently, most tumor antigens have been identified by expression cloning using serum, T-cell lines, or clones from cancer patients (6,7). Mass spectrometric techniques have also been used to identify peptides eluted from tumor cells (8). Although these approaches have identified a panel of genes that are immunologically relevant and may be used as potential cancer vaccines, these techniques require tremendous effort to identify a limited number of antigens. The success of these methods depends on the availability of clinical reagents (antibodies, T cells, and autologous tumor cell lines) and the presence of preexisting immune responses to tumor antigens. Suitable reagents often are not available, since patients are commonly immune compromised and because most

epithelial cancers are poorly immunogenic. Many antigens identified using these approaches are not cancer- or tissue-specific. Additionally, the antigens discovered via these methods are often not shared antigens, thus their expression is too restricted to permit wide clinical applicability and too irrelevant to the oncogenic process to prevent the selection of antigen-loss tumor mutants. Serological screening presents additional limitations, since the expression of genes in *Escherichia coli* may result in an inability to detect epitopes that are dependent on mammalian processing and modification systems. Finally, none of the above techniques provide a complete, systematic, and reliable comparison of gene expression differences between tumor and normal tissue types.

4. GENOMIC APPROACHES FOR ANTIGEN DISCOVERY

4.1. *Antigen Identification Via Genomic Approaches*

Revolutionary advances in genomic approaches have heralded the arrival of an exciting new era of antigen discovery. A new generation of genomic tools is being developed and utilized that is broadening our understanding of tumor biology and facilitating the identification of novel markers for cancer diagnosis and therapy. A burgeoning repertoire of genome-wide analysis techniques such as chromosome painting (9), comparative genomic hybridization (10), representational difference analysis (11), restriction landmark genome scanning (12), and high-throughput analysis of loss of heterozygosity (LOH) (13) have been used to study genome aberration in human cancers. Differential gene expression–profiling techniques, such as expressed sequence tag (EST) sequencing (14,15), serial analysis of gene expression (SAGE) (16,17), differential display PCR (DDPCR) (18), nucleic acid subtraction (19,20), and expression microarray analysis (21), are providing a wealth of new data that are being utilized to identify a panel of tumor- and tissue-specific or overexpressed genes that can serve as potential candidates for cancer diagnosis and therapy.

Once candidate genes are identified using gene expression profiling, additional steps can be integrated to evaluate the potential of these genes to serve as tumor antigens. These steps include further molecular characterization to confirm specificity and to identify full-length cDNA sequences; immunohistochemical methods to demonstrate the tumor- or tissue-specificity of a tumor antigen at the protein level and to determine the localization and extent of distribution of an antigen at the subcellular, cellular, and tissue levels; functional studies to elucidate the role of these antigens in tumorigenesis; and immunological studies to determine the antigenicity and immunogenicity of these antigens and to identify naturally processed epitopes that can be used as immunogens or reagents for monitoring antigen-specific immune responses in subsequent vaccine strategies.

4.2. *Pros and Cons of the Genomic Approach in Antigen Identification*

Using differential gene expression-profiling techniques to identify tumor antigens carries several advantages over alternative approaches using immune reagents from patients. First, since the focus of gene expression-profiling techniques is to discover tumor- or tissue-specific genes, the genes identified meet the specificity criteria; second, multiple tumor samples including tissues from metastatic lesions can be surveyed to identify genes that are broadly expressed in multiple tumor samples; third, because gene expression-profiling techniques can be rapid, efficient, and thorough, they can be used to systematically compare the cancer genome against the normal-tissue genome to iden-

tify genes that are differentially expressed between the two cell types; fourth, the gene expression-profiling approach does not depend on the availability of patient immunological reagents or strong preexisting immune responses. Thus, this method of inquiry provides a unique and important alternative strategy for the identification of tumor antigens. This is extremely important in tumor antigen discovery for cancers other than melanoma where cancer immunogenicity is weak and it is difficult to use immunological reagents for cancer antigen identification.

Although the genomic approach provides a number of significant advantages over immunological methods, several types of tumor antigens may potentially be overlooked, including genes with point mutations, genes encoded by alternative open reading frames, and genes expressed at a very low level. However, these three groups of antigens might not present useful vaccine targets anyway because they are often either too restrictedly or too rarely expressed. Nevertheless, another set of genes potentially missed through gene expression-profiling methods exists that might provide viable targets for a tumor vaccine. This group includes genes that are posttranscriptionally or posttranslationally modified, and as a consequence exhibit mRNA transcript levels that do not correlate with protein expression levels. An additional disadvantage of the gene expression-profiling approach lies in the fact that the genes identified may not be functionally or immunologically relevant to cancer. New and innovative strategies are being actively developed to address these issues including high-throughput functional screening assays to determine gene functions and their roles in tumorigenesis, immunological validation processes to determine the immunogenicity of genes, and protein-based discovery approaches to identify overexpressed genes that may not be reflected at the mRNA level.

4.3. Subtraction

Nucleic acid subtraction and microarray are among the most commonly used gene expression–profiling techniques. Several types of nucleic acid subtraction approaches have been employed that utilize slightly different protocols. The principle mechanism is to clone differences between two mRNA populations by hybridizing cDNA (mRNA) from one population (tester) to an excess amount of cDNA (mRNA) from another population (driver) and removing the common, hybridized sequences by PCR (11,20), enzyme digestion (22), or physical separation (19,23), thereby enriching for sequences unique to the tester population (19). The most commonly used subtraction technique is suppression subtractive hybridization (SSH), also known as PCR-based cDNA subtraction, which was developed by Diatchenko et al. (20). This procedure is based primarily on suppression PCR and combines normalization and subtraction in a single protocol. The normalization step equalizes the abundance of cDNAs and the subtraction step excludes the common sequences between the tester and driver populations. As shown in Fig. 1, the first step of the SSH protocol involves the synthesis of tester and driver double-stranded cDNAs from the two mRNA samples being compared. The tester and driver cDNA populations are then separately digested with the restriction endonuclease Rsa I to obtain shorter, blunt-ended molecules. Following Rsa I digestion, an adapter ligation step is performed in which two tester cDNAs are ligated with different adapters. Each adapter-ligated tester is then hybridized with an excess of driver cDNA that does not contain an adapter molecule. During this first hybridization step, the hybridization kinetics lead to equalization and enrichment of differentially expressed sequences. A second hybridization step is then performed in which the two samples from the first hybridization are

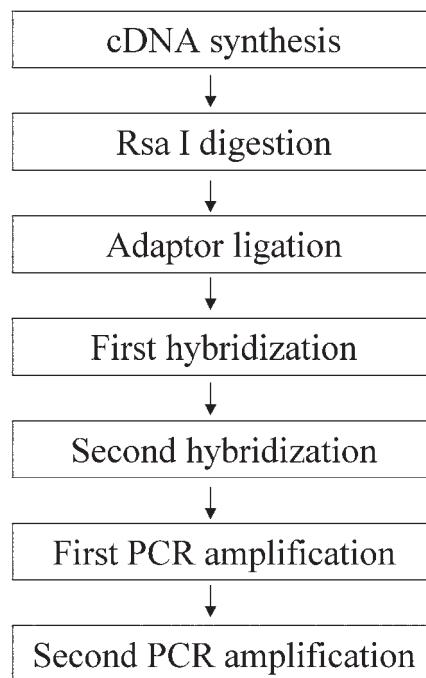


Fig. 1. Overview of suppression subtractive hybridization procedure.

mixed together and hybridized. Templates for PCR amplification are generated from differentially expressed sequences during this second hybridization step. Using suppression PCR, only differentially expressed sequences are amplified exponentially during the first PCR amplification. Figure 2 illustrates the mechanism of suppression PCR. Suppression occurs when complementary sequences are present on each end of a single-stranded cDNA. During each primer-annealing step, the hybridization kinetics strongly favor the formation of a pan-like secondary structure (overannealing of the shorter primers) thus preventing primer annealing. When occasionally a primer anneals and is extended, the newly synthesized strand will also have the inverted terminal repeats and form another pan-like structure. Thus, during PCR, the nonspecific amplification of sequences that are abundant in tester and carry the same adapters at both ends is efficiently suppressed. Specific amplification of cDNA molecules that are differentially expressed and carry different adapters at both ends can proceed normally. A second round of PCR amplification is required to further enrich differentially expressed sequences. Using this protocol, in a model system, Diatchenko et al. reported that the use of the SSH technique enabled an over 1000-fold enrichment of rare sequences in one round of subtractive hybridization (20). Indeed, the subtracted cDNA libraries generated by SSH technology contain an increased number of differentially expressed genes compared to most other types of subtraction approaches (24).

In our effort to identify vaccine targets, we have also employed a cDNA library subtraction technique that involves the construction of cDNA libraries from driver and tester mRNAs, the labeling of the driver cDNA library with biotin, the hybridization of driver and tester cDNA libraries, the removal of common hybridized sequences with

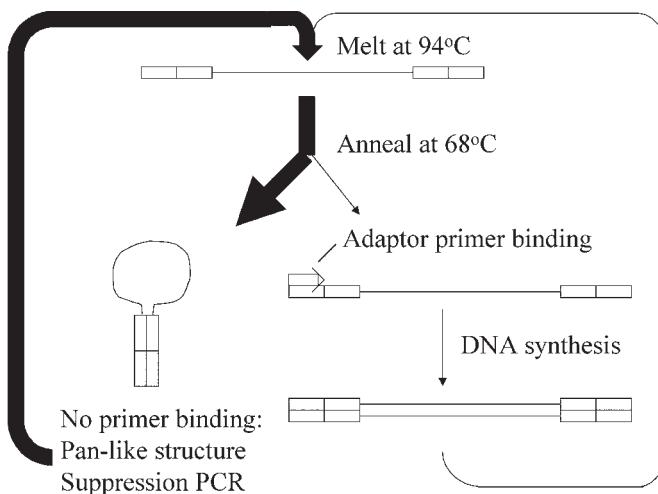


Fig. 2. Suppression PCR.

streptavidin, and phenol/chloroform extraction followed by the subsequent cloning of tester-specific genes into a cloning vector (19,25,26). Using this method, we demonstrated that cDNA library subtraction efficiently and effectively enriched for prostate tissue- and tumor-specific genes.

4.4. DNA Microarray

High-density DNA microarrays allow the simultaneous monitoring of hybridization signals of thousands of sequences in a single assay. Gene expression profiling is one of the most widespread applications of this technology. The interplay between the different processes used in manufacturing arrays, the various substrates upon which arrayed material can be deposited, and the types of material used for labeling cDNA probes, leads to many variations in the overall method employed to perform an individual microarray experiment. Two major platforms are in common use based on the process of manufacturing microarrays. The first involves oligonucleotides made by a photolithographic process (27) or an ink-jet oligonucleotide synthesizer (28). The second utilizes robotic deposition or “spotting” of DNA molecules (21). Spotted arrays are commonly referred to as “cDNA microarrays,” although cDNA clones, PCR products, or oligonucleotides can all be spotted. Depending on the substrate being used for microarrays, either silicon arrays or nylon membrane arrays are commonly used. The cDNA used for hybridizing sequences deposited on a microarray can be labeled with either fluorescence reagents or radioactive material. The use of fluorescence reagents permits the simultaneous hybridization of two separate cDNA populations labeled with two different fluorescence dyes in the same experiment. This competitive hybridization allows a direct comparison of the relative mRNA abundance between two populations. In contrast, radioactively labeled material is typically used with only a single cDNA sample for each experiment. Numerous computational tools have been developed to facilitate array scanning, image processing, data normalization, and analysis of huge amount of information from microarray experiments. More recently, clustering analysis tools have been developed for gene expression pattern studies (29–31).

Once a particular microarray platform has been determined, the selection of sequences to be arrayed and probes to be used for interrogating a given array are extremely important to the success of a microarray experiment. It can be very inefficient to use non-normalized or nonsubtracted libraries as the source of clones for microarray deposition, since most cDNA clones in these libraries come from ubiquitously expressed genes or genes having no connection to the disease process. On the other hand, arrays constructed from a limited number of preselected genes will represent only a small fraction of the human genome. Consequently, such an array will be neither comprehensive nor systematic. Other issues associated with microarray analysis include the use of cell line RNAs as probes that may not reflect natural biological processes, and the use of an insufficient number of probes that fails to adequately represent a suitable variety of cell types and tissues from different organs in the human body. Recent advances brought about by the completion of the human genome project have resulted in the ability to generate microarrays containing most of the ESTs. Prefabricated microarray chips are commercially available that contain genes from the entire analyzed human genome, as well as genes related to specific biological pathways or functional groups. Even so, a significant number of human genes remain undiscovered and, as a consequence, these types of microarrays will lack a subset of genes, particularly those that may be relevant to the disease process.

In order to address the limitations of using commercial microarrays, our lab has focused primarily on the development of custom microarrays. These custom arrays contained cDNA populations selected from subtracted libraries generated from primary or metastatic tumor samples. mRNA extracted from a broad spectrum of tumor and normal tissues from cancer patients and healthy individuals were used to determine the tissue distribution of the arrayed genes. The significant modifications incorporated into the custom microarray experiments have enabled us to interrogate the human tumor genome thoroughly and systematically and have led to the identification of a diverse set of novel and known genes.

4.5. Combining Subtraction and Microarray

The utilization of subtraction methods in combination with high-throughput microarray technology has proven to be an efficient and powerfully effective means of identifying either tumor- or tissue-specific genes. Subtraction protocols can be designed to enrich for tumor-specific, tissue-specific, or both tumor- and tissue-specific genes. In our attempts to discover prostate cancer antigens, we have performed experiments to target each of these specific gene groups (25). Subtraction of prostate tumors (tester) with normal prostate tissues (driver) is designed to enrich for prostate cancer-specific genes. Subtraction of normal prostate tissues (tester) with normal nonprostate tissues enriches for prostate tissue-specific genes. To target the recovery of both prostate tumor- and tissue-specific genes, subtraction was done by subtracting prostate tumors (tester) with normal nonprostate tissues (driver) (25). Table 1 summarizes several cDNA library subtractions we have performed in prostate cancer. Normal pancreas was used in several subtractions as the driver control because both prostate and pancreas are secretary organs with glandular structures. Use of normal pancreas as driver should be effective in eliminating genes shared by both tissues. More recently, pools of normal tissues, especially those that share similar cellular components with prostate tumors, have been used to eliminate nonspecific clones more efficiently. In addition, normal lymphoid cell mRNA

Table 1
Subtraction Library Summary

<i>Subtraction Library</i>	<i>Tester</i>	<i>Driver</i>	<i>Spiking^c</i>	<i># Clones on Microarray</i>
Subtraction 1	PT ^a	Normal pancreas	None	92
Subtraction 2	PT	NP ^b	None	122
Subtraction 3	NP	Normal pancreas	None	120
Subtraction 4	PT pool	Normal pancreas	Yes	114
Subtraction 5	PT	Normal pancreas	Yes	78

^aProstate tumor.

^bNormal prostate.

^cSpiking was done with PSA, human glandular kallikrein-1 (HGK-1), and cytochrome C oxidase subunit II (CCOII).

has been added to the driver to remove the immune system-related genes contributed by the infiltrating lymphocyte contamination in tumor samples.

In addition to prostate tumor, efforts have been made by our group to identify tumor antigens in a number of other cancer types as well, including breast, lung, and colon cancers. For each tumor type, multiple subtractions were performed and each subtraction was carried out utilizing a pool of multiple tumor samples as tester to increase the probability that genes expressed in defined subsets of tumors would be identified. To target genes of varying abundance, the ratio of tester to driver can be adjusted. In addition, cDNAs from abundant or known genes can be spiked into the driver, thereby reducing the chance of rediscovering these genes repeatedly. This greatly enhances the likelihood of identifying novel and less abundant genes. Also, colonies from subtracted libraries can be prescreened with redundant and previously identified genes to eliminate multiple copies of these cDNAs from being printed on microarrays, again increasing the chance of discovering novel sequences. By evaluating only a few hundred to a few thousand clones from a subtracted library (as opposed to millions of clones from a nonsubtracted library), it is possible to analyze the cancer genome of a given tumor type systematically and thoroughly to identify overexpressed genes.

In our discovery efforts, both PCR-select subtraction and cDNA library subtraction techniques have been utilized. A common set of genes has been identified by both techniques; however, each subtraction technique also revealed a unique, nonoverlapping subset of genes. The individual properties of the two subtraction techniques complement each other and by employing both, we were able to identify a wider spectrum of tumor- or tissue-specific genes. In prostate cancer, we believe that we have reached a high level of saturation based on the level of redundancy we have achieved in rediscovering more than 90% of previously isolated genes and based on the identification of almost all published prostate-tumor or tissue-specific genes. Included among the overexpressed novel genes or genes with no previously known association to cancer that we have identified by combining subtraction and microarray are Prostein (26), kallikrein 4 (KLK4), and P504S (α -methylacyl-CoA racemase, AMACR) (25) in prostate cancer; B726P (NY-BR-1), GABA_A receptor π subunit, and B709P in breast cancer (24); and L552S (an alternatively spliced isoform of XAGE-1) (32), L530S (a p53 homologue), and L531S

(a novel serine proteinase inhibitor) (33) in lung cancer. The gene expression profiles for all of these genes have been independently confirmed by additional methods including quantitative real-time PCR assays and Northern blot analyses. Protein expression has been demonstrated by immunohistochemical staining using antibodies developed against these gene products.

5. IMMUNOLOGICAL VALIDATION OF TUMOR ANTIGENS

Specificity is a prerequisite for an ideal tumor antigen, yet not the only requirement. The ability of a tumor antigen to elicit a strong immune response as well as the presence of multiple naturally processed immunogenic epitopes are also very important characteristics for an effective tumor antigen. In order to assess the immunogenicity of potential tumor antigens discovered by subtraction and microarray, we have utilized three different immunological validation approaches. The first involves the priming of naïve human T cells with either peptides or whole gene. Hural et al. has described the identification of naturally processed CD4 T-cell epitopes from the prostate-specific antigen, KLK4, using peptide-based in vitro stimulation (34). KLK4 is a serine protease and a member of the tissue kallikrein gene family that was discovered by multiple laboratories including our group, which identified KLK4 using subtraction and microarray approaches. The demonstration that KLK4-specific CD4 T cells exist in the peripheral circulation of normal male donors and the identification of naturally processed KLK4-derived CD4 T-cell epitopes support the use of KLK4 in vaccine strategies against prostate cancer. Prostein is another prostate tissue-specific protein that is highly and specifically expressed in normal and malignant prostate tissues (26). Using an in vitro whole-gene stimulation protocol, Prostein-specific CD8 T cells and Prostein-derived, naturally processed, class I restricted epitopes have been demonstrated in the peripheral T-cell repertoire of healthy male donors.

The second immunological validation approach utilized HLA-A2Kb transgenic mice for immunization with candidate genes identified by subtraction and microarray. Following immunization, both CD8 T-cell and antibody responses are evaluated to determine the quality and magnitude of immune responses against candidate antigens. Potential A2-restricted T-cell epitopes can be identified with this approach. These epitopes provide valuable tools for the development of peptide-based vaccination strategies against prostate cancer and for monitoring antigen-specific responses in vaccinated patients. Meagher et al. have evaluated the naked DNA, adenovirus, and DNA-adenovirus prime-boost immunization strategies for eliciting Prostein-specific CD8 T cells and antibody responses in HLA-A2Kb transgenic mice. Prostein-specific CD8 T-cell and antibody responses were induced by these immunization strategies, thus demonstrating the immunogenicity of Prostein in HLA-A2Kb transgenic mice. Multiple A2-restricted Prostein-derived T-cell epitopes were identified in the A2Kb transgenic mice studies.

The third approach involves examining prostate cancer patients for the presence of antibody responses against candidate antigens. Both peptide- and protein-based enzyme-linked immunoabsorbent assays (ELISAs) have been used to determine the levels of antigen-specific antibody responses in patient serum. The results were further confirmed by Western blot analysis using recombinant proteins. Using these methods, Day et al. have demonstrated the presence of KLK4-specific antibodies in the sera of 7/20 prostate cancer patients but not in any of the 13 sera from normal donors. The presence of naturally

occurring antigen-specific antibody responses in prostate cancer patients indicates that the candidate antigens are immunogenic in humans, thus allowing the induction of strong immune responses against tumors in vaccine strategies.

6. CONCLUDING REMARKS

The rapid development of powerful new techniques such as subtraction and microarray are providing new promise for the discovery of tumor antigens. The continuing advances in adjuvant and delivery systems to generate and expand strong immune responses to these tumor antigens will hopefully allow these proteins to be developed as an effective cancer-specific vaccine. Clinical trials are underway to test the effectiveness of these antigens in cancer patients and the results from these trials will undoubtedly give us insight regarding the utility of these second-generation tumor antigens.

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4

Tumor Antigen Discovery With T Cells

Paul F. Robbins, PhD

CONTENTS

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1. INTRODUCTION

Studies first carried out in the early 1980s demonstrated that incubation of tumor-infiltrating lymphocytes (TILs) with high doses of interleukin-2 (IL-2) resulted in the generation of CD8+ cytotoxic T cells (CTL) that recognized tumor cells in a major histocompatibility complex (MHC)-restricted manner. This procedure resulted in the generation of tumor-reactive T cells from about 50% of patients with melanoma and a smaller percentage of patients bearing other malignancies. The *in vitro* sensitization of peripheral blood mononuclear cells (PBMCs) from cancer patients with autologous or human leukocyte antigen (HLA)-matched allogeneic tumor cells in mixed lymphocyte tumor cultures (MLTCs) also resulted in the generation of tumor-reactive T cells. These cells as well as the tumor-reactive TIL-lysed tumor and released a variety of cytokines including interferon- γ (IFN- γ), granulocyte macrophage colony-stimulating factor (GM-CSF), or tumor necrosis factor- α (TNF- α) in response to human leukocyte antigen (HLA)-matched tumor targets. Tumor-reactive class II restricted T cells have also been identified in a significant percentage of the cultures of sensitized PBMC or TIL cultures containing class I-restricted tumor-reactive T cells.

These observations led to efforts to clone the genes that encode these antigens. A transient transfection technique that had previously been used to identify several antigens recognized by mouse tumor-reactive T cells was utilized in 1991 to identify the first human tumor antigen recognized by T cells, now termed *MAGE-A1* (1). In subsequent studies, a variety of techniques have been used to identify tumor antigens that are

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expressed in melanoma as well as other tumor types. These antigens have been isolated using primarily genetic techniques, but additional methods that have been utilized include elution of peptides from MHC molecules and the purification of whole-protein antigens from tumor cells. The use of patient sera to screen tumor cell cDNA expression libraries, a technique that has been designated SEREX (serological analysis of gene expression), has also resulted in the identification of antigens that are expressed on a variety of tumor types. Although many of the antigens isolated using this technique appear to represent widely expressed auto-antigens, a subset of these antigens has now been shown to correspond to antigens that have been identified using screening assays carried out using tumor-reactive T cells.

Several broad categories of antigens have been identified using these techniques. The family of melanocyte differentiation antigens are also expressed in normal skin melanocytes and pigmented retinal epithelial cells, but their expression in tumor cells is limited to melanomas. A wider expression pattern has been observed for the cancer testis (CT) family of antigens, which are expressed in melanomas as well as a variety of common epithelial tumors such as prostate, lung, and breast carcinomas. Expression of CT antigens in normal tissues is limited to normal testis cells. Certain antigens appear to be expressed in a wide variety of normal tissues; however, the levels of expression in these tissues are generally lower than those found in tumor cells. Finally, antigens that are encoded by gene products that are expressed exclusively in tumor cells have also been identified. These include transcripts derived from mutated genes as well as a relatively small number of transcripts that are not expressed at any significant level in normal tissues. This chapter will discuss the methods of identifying tumor antigens using T cells in melanoma, which has been the most extensively studied tumor and consequently has also been where most of the T-cell-based approaches were developed. The same techniques are also being applied successfully to identify antigens in other tumors.

Identification of the specific peptide epitope recognized by tumor-reactive T cells has been aided by the definition of peptide-binding motifs for multiple HLA class I and class II alleles (2). Minimal antigenic peptides generally are composed of between 8 and 10 amino acids, although longer peptides have been found to be required for optimal recognition of certain HLA class I peptide epitopes. Particular amino acids have been shown to predominate at the 2 or 3 positions within these peptides, which have been designated the anchor amino acids. For example, peptides that bind to the HLA-A2 class I gene product generally possess an aliphatic residue such as L or M at position 2 (P2) and a V or L residue at the carboxy terminus of the peptide (P9 or P10). The binding affinity of individual peptides to class I MHC molecules appears generally to be predominantly influenced by the amino acids present at these positions, although residues with a less significant impact on peptide binding, termed secondary anchors, have also been identified for many alleles. The HLA class II peptide-binding pocket has a more open structure than the class I binding pocket, and as a result the amino and carboxyl termini of class II binding peptides are less well defined than class I binding peptides. Nevertheless, binding motifs that have been defined for several HLA class II molecules contain a core region of approx 10 amino acids that contains two or three anchor positions. Use of the peptide-binding motifs, in conjunction with peptide-binding assays, has led to a number of studies in which candidate peptides identified using these techniques have then been used to generate specific T cells from PBMCs derived from normal individuals or cancer patients. Although a general correlation has been noted between the peptide affinity and

the ability to generate specific T cells (3), many of the peptides that are naturally recognized by tumor-reactive T cells have been found to bind to specific HLA alleles with relatively low affinities, which results from the presence of a nonoptimal residue at one of the primary anchor positions. Substitution of optimal for nonoptimal anchor residues has been shown to enhance the immunogenicity of certain peptides. One potential explanation for these observations is that self-tolerance can play a role in shaping the T-cell repertoire, and evidence supporting this hypothesis has been obtained by several investigators.

2. ANTIGENS RECOGNIZED BY HLA CLASS I-RESTRICTED T CELLS

2.1. *Cancer Testis Antigens*

The *MAGE-A1* gene is a member of a large family of genes that are limited in their expression in normal tissues to the testis and placenta. These cancer/testis antigens appear to comprise at least 10 gene families, and the *MAGE-A1* gene appears to be a representative of the largest family of cancer/testis genes, which contains over 25 genes (4). A large number of antigens have been found to be encoded by members of these gene families (Table 1). Individual members of these families are expressed in up to 60% of melanoma, breast, lung, bladder, and prostate cancers. Homologous regions of the *MAGE-A1* and *MAGE-A3* genes have been found to encode the dominant HLA-A1-restricted epitopes that are derived from these proteins (5). An HLA-A2 peptide derived from the *MAGE-A3* protein was initially reported to be capable of generating peptide-reactive T cells as well as tumor-reactive T cells (6). In a subsequent study, however, it appeared that T cells generated with this peptide could recognize peptide-pulsed target cells but failed to recognize tumor cells or HLA-A2-positive target cells that were transfected with the *MAGE-A3* gene (7). Treatment of tumor cells with the proteosomal inhibitor lactacystin appeared to sensitize those cells for lysis, indicating that degradation of this peptide by proteases within tumor cells may generally be responsible for the failure of T cells to recognize these targets.

The cancer/testis antigen NY-ESO-1 was initially identified following the screening of a λ -phage cDNA expression library with serum from a patient with esophageal squamous cell carcinoma (8), and it now appears that over 50% of patients bearing NY-ESO-expressing tumors possess significant titers of anti-NY-ESO-1 antibodies (9). In contrast, antibodies directed against the majority of tumor antigens that have been described are only rarely detected in cancer patients. In addition, reduced titers of anti-NY-ESO-1 antibodies in several patients that were examined appeared to be associated with the regression of bulky disease, indicating that antigen load may play a role in maintaining anti-NY-ESO-1 antibody responses. A T-cell line that was derived from a patient that possessed a high titer of anti-NY-ESO-1 antibodies was subsequently found to recognize NY-ESO-1 in the context of the HLA-A2 class I gene product (10). Screening of peptides derived from this protein revealed that a peptide comprising amino acids 157 to 167, (NY-ESO-1_{157–167}), as well as two overlapping peptides, NY-ESO-1_{157–167} and NY-ESO-1_{155–163} were recognized by the T-cell line. Subsequent experiments demonstrated that, whereas the NY-ESO-1_{157–165} peptide binds with relatively low avidity to the HLA-A2 molecule, target cells pulsed with this peptide are nevertheless recognized well by tumor-reactive T cells (11). The NY-ESO-1_{157–167} and NY-ESO-1_{155–163} peptides, though capable of binding HLA-A2 with a significantly higher avidity than the NY-ESO-1_{157–165}

Table 1
HLA Class I-Restricted Epitopes of Cancer/Testis Antigens

<i>Gene</i>	<i>HLA Allele</i>	<i>Epitope</i>
<i>MAGE-1</i>	A1	EADPTGHSY
<i>MAGE-1</i>	A3	SLFRAVITK
<i>MAGE-1</i>	A28	EVYDGREHSA
<i>MAGE-1</i>	B53	DPARYEFLW
<i>MAGE-1</i>	Cw2	SAFPPTINF
<i>MAGE-1</i>	Cw3	SAYGEPRKL
<i>MAGE-1</i>	A24	NYKHCFCPEI
<i>MAGE-1</i>	Cw16	SAYGEPRKL
<i>MAGE-2</i>	A2	KMVELVHFL
<i>MAGE-2</i>	A2	YLQLVFGIEV
<i>MAGE-2</i>	A24	EYLQLVFGI
<i>MAGE-3</i>	A1	EVDPIGHLY
<i>MAGE-3</i>	B*3501	EVDPIGHLY
<i>MAGE-3</i>	A2	FLWFPRALV
<i>MAGE-3</i>	A24	TFPDLESEF
<i>MAGE-3</i>	B44	MEVDPIGHLY
<i>MAGE-6</i>	A34	MVKISFFPR
<i>MAGE-A4</i>	A2	GVYDGREHTV
<i>MAGE-A10</i>	A2	GLYDGMEHL
<i>MAGE-12</i>	Cw*0702	VRIGHLYIL
<i>BAGE</i>	Cw16	AARAVFLAL
<i>DAM</i>	A2	FLWGPRAYA
<i>GAGE-1/2</i>	Cw6	YRPRPRRY
<i>RAGE</i>	B7	SPSSNRIRNT
<i>NY-ESO-1</i>	A2	SLLMWITQCFL
<i>NY-ESO-1</i>	A2	SLLMWITQC
<i>NY-ESO-1</i>	A2	QLSLLMWIT
<i>NY-ESO-1</i>	A31	ASGPGGGAPR
<i>NY-ESO-1</i>	A31	LAAQERRVPR
<i>NY-ESO-1/LAGE</i>	A2	MLMAQEAL AFL

peptide, appeared to only weakly stimulate these T cells. Thus, it appears that NY-ESO-1_{157–165} may represent the peptide that is naturally processed and presented on the surface of tumor cells. The NY-ESO-1_{157–165} peptide contains a cysteine residue at the carboxy terminus, and substitution of amino acids such as alanine or valine for this residue appeared to further enhance recognition by CD8+ T cells, presumably by inhibiting the formation of peptide dimers that are linked through a disulfide bond.

Additional HLA class I-restricted epitopes derived from NY-ESO-1 and LAGE-1, a gene product that shares over 95% amino acid similarity with NY-ESO-1, as well as other members of the cancer/testis family have now been identified. Tumor-reactive CTL clones derived from TILs have been found to recognize an epitope derived from the normal NY-ESO-1 protein in the context of the HLA-A31 class I allele, whereas a second NY-ESO-1-reactive, HLA-A31-restricted T-cell clone was found to recognize a peptide

that resulted from the translation of an alternative open reading frame (ORF) of the NY-ESO-1 transcript (12). This represents a novel and somewhat unexpected observation, but additional examples where a single transcript encodes two protein products have now been described. The *LAGE-1* gene was recently found to encode a CD8 epitope derived from the amino terminus of an alternative open reading frame (13), and products derived from the alternative ORFs of additional gene products have also been shown to be recognized by tumor-reactive T cells, as described further below. The BAGE (14) and GAGE (15) family of genes that were identified following the screening of cDNA libraries with tumor-reactive T-cell clones appear to represent additional examples of cancer/testis genes that encode tumor antigens.

2.2. Melanocyte Differentiation Antigens

The family of melanocyte differentiation antigens includes a number of gene products, such as gp100, TRP-1, TRP-2, and tyrosinase, that are involved in the synthesis of melanin (Table 2). Other molecules identified as tumor antigens that may play a role either in melanin synthesis or the biogenesis of melanosomes include MART-1/Melan-A (16,17) and AIM-1 (18).

In order to identify the T-cell epitopes derived from these antigens, peptides that conformed to the HLA-A2-binding motif were synthesized and tested for their ability to sensitize target cells for recognition by antigen-reactive T cells derived from TILs or from PBMC cultures stimulated with tumor cells. Among a panel of HLA-A2-restricted TILs with specific tumor cell reactivity, nearly all recognized MART-1, indicating that this represents an immunodominant antigen in HLA-A2 melanoma patients (19). All MART-1-reactive TILs appear to recognize the MART-1:27–35 nonamer, as well as overlapping peptides extended by one additional amino acid at either end of this peptide (20).

The melanocyte differentiation antigen gp100 also appears to be immunodominant in HLA-A2-positive melanoma patients (21,22). Three gp100 epitopes, gp100:209–217 (ITDQVPFSY), gp100:280–288 (YLEPGPVTA), and gp100:154–162 (KTWGQYWQV), are frequently recognized by HLA-A2-restricted TILs (19). In another study, five HLA-A2-restricted T-cell lines generated from PBMCs by in vitro sensitization with tumor cells were tested for their ability to recognize peptides that had been fractionated using reversed phase high-performance liquid chromatography (HPLC) columns (22). Sequencing of peptides in positive fractions through the use of tandem mass spectrometry resulted in the identification of the gp100:280–288 peptide as an antigen that was recognized by each of the five HLA-A2-restricted T-cell lines that were tested. Several additional gp100 epitopes recognized by melanoma-reactive T cells in the context of HLA-A2 (23), as well as HLA-A3 (24), HLA-A24 (25), and HLA-Cw8 (26), have been identified. Multiple epitopes recognized by HLA-A2-restricted tyrosinase (27) and TRP-2 (18,28) reactive T cells have also been identified.

The HLA-binding motifs have been used to identify additional epitopes from previously identified tumor antigens. These motifs have been used in conjunction with class I HLA-binding assays to identify candidate epitopes from tissue-specific proteins as well as proteins that are overexpressed in tumor cells. Autologous PBMCs or dendritic cells that have been pulsed with these peptides have then been used to carry out multiple in vitro stimulations to generate peptide-reactive T cells. Using this approach, candidate peptides from gp100 (29), tyrosinase (30), and TRP-2 (28) have been used to generate T cells that appear to recognize unmanipulated tumor cells.

Table 2
HLA Class I-Restricted Epitopes of Melanocyte Differentiation Antigens

<i>Gene</i>	<i>HLA Allele</i>	<i>Epitope</i>
<i>MART-1</i>	A2	AAGIGILTV
<i>MART-1</i>	A2	EAAGIGILTV
<i>MART-1</i>	A2	AAGIGILTVVA
<i>MART-1</i>	A2	ILVILGVVL
<i>MART-1</i>	B45	AEEAAGIGILT
<i>MART-1</i>	B45	AEEAAGIGIL
<i>MCIR</i>	A2	TILLGIFFL
<i>MCIR</i>	A2	FLALIICNA
<i>Gp100</i>	A2	KTWGYWQV
<i>Gp100</i>	A2	ITDQPFSV
<i>Gp100</i>	A2	YLEPGPVTA
<i>Gp100</i>	A2	LLDFTATLRL
<i>Gp100</i>	A2	VLYRYGFSV
<i>Gp100</i>	A2	RLMKQDFSV
<i>Gp100</i>	A2	RLPRIFCSC
<i>Gp100</i>	A3	LIYRRRLMK
<i>Gp100</i>	A3	ALLAVGATK
<i>Gp100</i>	A24	VYFFLPDHL(intron)
<i>Gp100</i>	Cw8	SNDGPTLI
<i>Tyrosinase</i>	A1	KCDICTDEY
<i>Tyrosinase</i>	A1	SSDYVIPIGY
<i>Tyrosinase</i>	A2	MLLAVLYCL
<i>Tyrosinase</i>	A2	YMDGTMSQV
<i>Tyrosinase</i>	A2	CLLWSFQTSA
<i>Tyrosinase</i>	A24	AFLPWHRLF
<i>Tyrosinase</i>	B44	SEIWRDIDF
<i>TRP-1</i>	A31	MSLQRQFLR
<i>TRP-2</i>	A2	SYVYDFFFVWL
<i>TRP-2</i>	A2	YAIIDLPVSV
<i>TRP-2</i>	A31,A33	LLGPGRPYR
<i>TRP-2</i>	Cw8	ANDPIFVVL
<i>TRP-2</i>	A2	ATTNILEHV
<i>TRP-2</i>	A2	SLDDYNHLV
<i>AIM-1</i>	A2	AMFGREFCYA
<i>AIM-2</i>	A1	RSDSGQQARY
<i>OA-1</i>	A24	LYSACFWWL
<i>P.-polypeptide</i>	A2	IMLCLIAAV

Tissue-specific molecules that were not previously shown to contain T-cell epitopes have also been screened using the candidate epitope approach. Thirty-one candidate HLA-A2-binding peptides were initially identified from the melanocortin receptor (MC1R) through the use of the peptide-binding motif, and three of the peptides that were tested appeared to be capable of generating tumor-reactive T cells from melanoma patient PBMCs (31). Stimulation of PBMCs from melanoma patients with peptides from two proteins that are also expressed exclusively in cells of the melanocyte lineage, p. polypep-

Table 3
HLA Class I-Restricted Epitopes of
Widely Expressed Antigens

<i>Gene</i>	<i>HLA Allele</i>	<i>Epitope</i>
<i>PRAME</i>	A24	LYVDSLFL
<i>PRAME</i>	A2	VLDGLDVLL
<i>PRAME</i>	A2	SLYSFPEPEA
<i>PRAME</i>	A2	ALYVDSLFFL
<i>PRAME</i>	A2	SLLQHLIGL
<i>P15</i>	A24	AYGLDFYIL
<i>AIM-2</i>	A1	RSDSGQQARY
<i>BING-4</i>	A2	CQWGRLWQL

tide (32) as well as OA-1 (33), also gave rise to tumor-reactive T cells. When T cells derived from an individual with a knock-out of the OA-1 gene were utilized to carry out in vitro sensitizations, it appeared that it was easier to generate T cells than from normal donors, indicating a role for expression of this antigen in normal tissues in inducing a state of partial tolerance. Melanoma patients, however, appeared to have a higher frequency of T cells reactive with the OA-1 peptide than normal donors, indicating that the tumor could partially reverse this tolerant state.

2.3. Widely Expressed Gene Products

A variety of tumor antigens appear to be encoded by genes that are expressed at significant levels in normal tissues (Table 3). One potential explanation for these findings may be that the gene that was isolated encoded a cross-reactive epitope, but additional mechanisms also appear in some cases to influence antigen recognition. One of the antigens isolated using a melanoma-reactive T-cell clone, termed PRAME, appeared to be expressed at relatively high levels in normal adult testis as well as normal endometrium, ovary, and adrenal tissues (34). A variety of additional tumor types, including lung squamous carcinomas, adenocarcinomas, renal carcinomas, sarcomas, head and neck squamous cell carcinomas, and acute leukemias also appeared to express PRAME at relatively high levels. This T-cell clone recognized a recurrent tumor isolated in 1993 from the autologous patient that had lost expression of all HLA class I alleles with the exception of HLA-A24, MEL.B, but at the same time failed to recognize an earlier tumor cell line derived from the same patient. A tumor cell line isolated in 1988 that expressed all of the expected HLA class I alleles, MEL.A, was not recognized by the T-cell clone. Engagement of the p58.2 NK inhibitory receptor present on PRAME-reactive T cells by the HLACw7 gene product that was expressed on the MEL.A tumors appeared to attenuate responses of PRAME-reactive T cells. Additional products that appear to be other examples of widely expressed products that are nevertheless recognized by tumor-reactive T cells have also been described (35,36), raising the possibility that additional mechanisms may be responsible for recognition of these products.

2.4. Tumor-Specific Antigens

Tumor-specific antigens generally arise either from mutations that occur within regions encoding T-cell epitopes or from transcripts whose expression appears to be strictly

Table 4
HLA Class I-Restricted Epitopes
of Tumor Specific Antigens

<i>Gene</i>	<i>HLA Allele</i>	<i>Epitope</i>
<i>CDK-4</i>	A2	ACDPHSGHFV
<i>β-catenin</i>	A24	SYLDSFIHF
<i>Caspase-8</i>	HLA-B35	FPSDSWCYF
<i>GnT-V (intron)</i>	A2	VLPDVFIRCV
<i>MUM-1</i>	B44	EEKLIVVLF
<i>MUM-2</i>	B44	SELFRSGLDY
<i>MUM-2</i>	Cw6	FRSGLDSYV
<i>Myosin-m</i>	A3	KINKNPKYK
<i>TRP-2 (intron)</i>	A68	EVISCKLIKR
<i>MART-2</i>	A1	FLEGNEVGKTY

limited to tumor cells (Table 4). Use of a T-cell clone that recognized a squamous cell carcinoma to screen a cDNA library resulted in the identification of a mutated Caspase-8 gene product (37). A nucleotide substitute in the normal stop codon resulted in extension of the normal open reading frame and generation of the T cell epitope. This mutation was observed in only one out of a large number of tumor cell lines, however, indicating that this was a relatively rare event. A mutated CDK4 gene product also encoded a CD8+ T-cell epitope, and the identical mutation was identified in 2 out of the 28 tumor cell lines that were analyzed (38). Binding to the HLA-A2 restriction element used for recognition of the CDK4 epitope was not altered by the mutation, and thus this mutation appeared to affect peptide binding to the T-cell antigen receptor. A mutated β-catenin gene product was identified by screening an autologous tumor cDNA library with an HLA-A24-restricted T-cell clone, and in this case the mutation resulted in the substitution of an optimal tyrosine for a non-optimal serine residue at the terminal anchor position in the T-cell epitope (39). Analysis of β-catenin gene products isolated from melanoma cell lines indicated that the identical mutation was present in tumors from 4 of the 46 patients that were analyzed (40; and Robbins, P., unpublished data).

A number of the mutations that have been identified as a result of screening with tumor-reactive T cells may also play a role in phenotypic alterations found in tumor cells. The mutation in the CDK4 gene product that is described above appears to alter the binding to p16 (INK4a), a protein that is frequently mutated in melanomas and that plays a role in the inhibition of the activity of the normal CDK4 gene (41). The normal caspase-8 gene product plays a critical role in apoptosis that is mediated by Fas and TNF (42,43), whereas the mutated gene appears to be attenuated in its ability to promote apoptosis. The mutated β-catenin gene product accumulated to abnormally high levels within tumor cells, leading to binding of TCF/LEF proteins and activation of genes that are involved in growth control such as cyclin D and c-myc (44). Additional melanoma cell lines were found to contain mutations within a region of the gene that encodes the original β-catenin HLA-A24 epitope, and many of these mutations also resulted in the accumulation of this protein, within the cell, which may have played a role in the loss of tumor-cell growth control.

A relatively small number of tumor-specific transcripts that do not appear to have arisen as a result of somatic or germline mutations have also been identified. Approximately 50% of all melanomas that were tested appear to express an aberrant transcript that initiated within an intron of the *N*-acetylglucosaminyltransferase V gene (45). The transcript was not expressed at significant levels in any of the human tissue samples tested including skin, which contain normal melanocytes, indicating that this may represent a tumor-specific transcript. An intronic sequence derived from an aberrant transcript of the TRP-2 gene was found to encode a T-cell epitope (46). Normal skin melanocytes and retinal tissue expressed the normal transcript but failed to express significant levels of the aberrant transcript, indicating that this gene may be expressed in a tumor-specific manner.

A tumor-specific product whose recognition is influenced by proteolytic processing mechanisms has also been described. In a recent study, a short 11 amino acid ORF that was found to encode a T-cell epitope appeared to be a product of a pseudogene that was similar to a portion of the 3' untranslated region of the homeoprotein HPX42B (47). A similar peptide that was derived from the 3' untranslated region of the normal HPX42B gene was recognized when pulsed on target cells expressing HLA-B*1302, but transfection of the normal HPX42B gene did not lead to T-cell recognition. The sequence that encoded the valine residue present at the carboxy terminus of the peptide epitope was followed by a stop codon in the pseudogene transcript, whereas this residue was followed by a tryptophan residue in the normal HPX42B gene product. Mutagenesis studies demonstrated that T-cell recognition was dependent on the presence of a stop codon at this position, indicating that expression of a product that did not require additional processing at the carboxy terminus may have compensated for the relatively low level of expression of this transcript.

The antigen FGF-5 was recently identified as a target of an HLA-A3-restricted, renal cancer-specific T-cell clone following the screening of an autologous tumor cell cDNA library (48). Analysis of the expression pattern of this gene product using quantitative RT-PCR assays revealed that a subset of renal as well as breast and prostate carcinomas expressed this product. Normal tissues did not appear to express significant levels of FGF-5, and expression of this gene product was correlated with T-cell recognition in cell lines that either naturally expressed HLA-A3 or were transduced with a retrovirus encoding HLA-A3. This antigen thus may represent an important immunotherapy target as only a relatively small number of tumor-antigen target molecules have been identified in renal, breast, and prostate carcinomas.

3. ANTIGENS RECOGNIZED BY TUMOR-REACTIVE, CLASS II-RESTRICTED T CELLS

Several of the antigens that are recognized by HLA class II-restricted, tumor-reactive T cells have been identified using approaches that are similar to those used to identify HLA class II-restricted antigens (Tables 5–7). In one group of studies, the transformed human kidney cell line 293 was transfected with genes that encoded the invariant HLA class II DR α and the appropriate DR β chain products along with genes that are involved with HLA class II antigen processing. Use of a cell line that expressed the HLA-DR β 1*0101 restriction element to screen an autologous melanoma cell cDNA library resulted in isolation of a gene that encoded a fusion protein resulting from a chromosomal translocation (49). Using the same approach, a product of the CDC27 gene containing a

Table 5
HLA Class II–Restricted Epitopes of Cancer/Testis Antigens

<i>Gene</i>	<i>HLA Allele</i>	<i>Epitope</i>
<i>MAGE-3</i>	DR β 1*11	TSYVKVLHHMVKISG
<i>MAGE-3</i>	DR β 1*13	AELVHFLLLKYRAR
<i>MAGE-1,2,3,6</i>	DR β 1*13	LLKYRAREPVTKAЕ
<i>MAGE-3</i>	DP β 1*0401,0402	TQHFVQENYLEY
<i>NY-ESO-1</i>	DR β 1*0401	PGVLLKEFTVSG
<i>NY-ESO-1</i>	DP β 1*0401,0402	SLLMWITQCFLPVF
<i>NY-ESO-1</i>	DR β 1*0401	PGVLLKEFTVSGNIL
<i>NY-ESO-1</i>	DR β 1*0401	LKEFTVSGNILTIRL
<i>NY-ESO-1</i>	DR β 4*0101-0103	PLPVPGVLLKEFTVSGNI
<i>NY-ESO-1</i>	DR β 4*0101-0103	VLLKEFTVSGNILTIRLT
<i>NY-ESO-1</i>	DR β 4*0101-0103	AADHRQLQLSISSCLQQL
<i>MART-1</i>	DR β 1*0401	RNGYRALNDKSLHVGTQCALTR

Table 6
HLA Class II–Restricted Epitopes of Melanocyte Differentiation Antigens

<i>Gene</i>	<i>HLA Allele</i>	<i>Epitope</i>
<i>Tyrosinase</i>	DR β 1*0401	QNILLSNAPLGPQFP
<i>Tyrosinase</i>	DR β 1*0401	DYSYLQDSDPDSFQD
<i>Gp100</i>	DR β 1*0401	WNRQLYPEWTEAQRLD
<i>Gp100</i>	DR β 1*0701	TGRAMLGTHTMEVTVYH
<i>TRP-1</i>	DR β 1*1501/1502	SLPYWNFATGKN
<i>TRP-2</i>	DR β 1*1501/1502	ALPYWNFATGRN

Table 7
HLA Class II Epitopes of Tumor-Specific Antigens

<i>Gene</i>	<i>HLA Allele</i>	<i>Epitope</i>
<i>CDC-27</i>	DR β 1*0401	FSWAMDLDPKGA
<i>LDLR-FUT</i>	DR β 1*0101	WRRRAPAPGAK
<i>TPI</i>	DR β 1*0101	ELIGILNAAKVPAD
<i>EphA3^a</i>	DR β 1*11	DVTFNIACKKCG

^aExpression of EphA3 in normal tissues appeared to be confined to tissues that do not constitutively express HLA class II molecules (70).

single point mutation resulting in the substitution of a leucine for a serine residue was also identified as the target of HLA-DR β 1*0401-restricted, tumor-reactive CD4+ TIL (50). Transfectants expressing the normal CDC27 gene product were not recognized by the tumor-reactive TIL, but paradoxically, the epitope did not appear to be encoded within the mutated portion of the transcript. The mutation in the CDC27 coding region, which resulted in a non-conservative substitution of a leucine for a serine residue, appeared to

redirect the protein from the nucleus to the cytoplasm in the tumor cell line. This alteration may thus have resulted in accumulation of the mutated CDC27 product in a cellular compartment where the protein could be more efficiently processed and presented on HLA class II products.

A variety of additional approaches have been used to identify HLA class II-restricted antigens. Using conventional protein purification methods, a mutated product of the triosephosphate isomerase gene was identified as the target of HLA-DR β 1*0101-restricted, tumor-reactive TIL (51). Tumor-reactive CD4+ T cells have also been tested for their ability to recognize antigens initially identified as targets of HLA class I-restricted T cells, presuming that many antigens may be recognized by both HLA class I- and class II-restricted T cells. In one study, EBV B cells expressing the appropriate HLA class II restriction element were pulsed with lysates of COS cells that had been transfected with genes encoding tyrosinase, gp100, TRP-1, or TRP-2. Using this approach, two epitopes of tyrosinase were shown to be recognized by an HLA-DR β 1*0401-restricted TIL culture (52). In addition, epitopes derived from nearly identical regions in the TRP-1 and TRP-2 proteins were found to be recognized by HLA-DR β 1*1502-restricted tumor-reactive CD4+ T-cell clones (53).

The in vitro generation of CD4+ T cells using candidate epitopes derived from previously described tumor antigens has also resulted in the identification of new HLA class II-restricted T-cell epitopes. Target cells that were either pulsed with candidate epitopes derived from known tumor antigens or with whole recombinant tumor antigen proteins have been used to carry out in vitro T-cell stimulation. In one study, antigen-presenting cells were pulsed with MAGE-A3 peptides that were predicted to bind to a variety of HLA-DR molecules and used to sensitize PBMCs from melanoma patients (54). Stimulation with three of the peptides that were tested resulted in the generation of peptide-specific T cells, and T cells that were elicited with one of these peptides appeared to react with target cells that were pulsed with the recombinant MAGE-A3 protein, as well as tumor cells that expressed the HLA-DR β 1*11 class II restriction element. In another study, dendritic cells that had been pulsed with recombinant MAGE-3 protein were used to stimulate PBMCs from autologous melanoma patients. Three T-cell clones that were generated from these cultures recognized protein-pulsed autologous EBV B cells as well as two overlapping but distinct MAGE-A3 peptides in the context of HLA-DR β 1*13 (55). A MAGE-A3 peptide epitope that was recognized in the context of HLA-DP4 (56) as well as an epitope of MAGE-A1 that was recognized in the context of HLA-DR15 (57) were also identified using this approach. Autologous dendritic cells have also been transduced with a retrovirus encoding gp100 and used to sensitize PBMCs from melanoma patients (58). Sensitized T cells that were generated from one of these patients recognized target cells pulsed with a previously undescribed epitope of gp100 in the context of the HLA class II allele DR β 1*0701 as well as tumor cells that naturally expressed these gene products.

Transgenic mice that express human HLA class II alleles have also been used to identify epitopes recognized by human tumor-reactive T cells. In one study, candidate peptides from the human TRP-1 antigen were screened in a transgenic mouse that expressed the HLA-DR β 1*0401 molecule (59). When one of the peptides that was identified using this method was used to sensitize human PBMCs in vitro, tumor-reactive T cells could readily be generated from patients with melanoma. The in vitro response of melanoma

patients appeared to be significantly enhanced relative to normal individuals, indicating a role for tumors in sensitizing T cells, as noted above for certain HLA class I-restricted antigens.

Multiple HLA class II-restricted epitopes of the NY-ESO-1 antigen have also now been identified. Immunization of transgenic mice expressing the human HLA-DR β 1*0401 gene product with the recombinant NY-ESO-1 protein resulted in the identification of multiple antigenic peptides that were then tested for their ability to stimulate human CD4+ T cells (60). Three of the eight peptides that were tested were capable of generating peptide-reactive T cells from human PBMC, and one of these peptides, NY-ESO-1₁₁₆₋₁₃₅, was found to efficiently generate tumor-reactive T cells. An overlapping peptide was subsequently found by other investigators to be immunogenic in HLA-DR β 1*0401-positive melanoma patients (61). In another study, three partially overlapping peptides from the same region of the NY-ESO-1 appeared to be recognized by class II HLA-DR β 4*01-restricted T cells (62). Overlapping peptides from the normal ORF of NY-ESO-1 and the closely related gene LAGE-1, as well as alternative ORFs derived from these products that were previously shown to encode epitopes recognized by class I-restricted tumor-reactive T cells were also used to generate T cells from melanoma patients. The results indicated that two peptides derived from an alternative ORF of LAGE-1-generated T cells that recognized a naturally processed peptide derived from this antigen (63).

The NY-ESO-1₁₆₁₋₁₈₀ peptide that was initially identified using HLA-DR β 1*0401-positive transgenic mice was also found to be immunogenic in melanoma patients (64). Curiously, T cells from an HLA-DR β 1*0401-positive patient that were generated using this peptide appeared to recognize allogeneic peptide-pulsed target cells that failed to express this class II allele. The results of antibody-blocking studies indicated that preincubation of target cells with antibodies directed against HLA-DP, but not HLA-DR or DQ products, blocked T-cell responses to peptide pulsed target cells. In addition, nearly all of the cell lines that were recognized following peptide pulsing expressed either the HLA-DP β 1*0401 or -DP β 1*0402 class II alleles which, in previous studies, were found to be expressed by between 43% and 70% of individuals (65). Seven out of the 8 NY-ESO-1-positive melanoma cell lines that were tested were recognized by T cells specific for the NY-ESO-161-180 peptide derived from patients that expressed the HLA-DP β 1*0401 class II allele, whereas the 1 melanoma line that was not recognized did not express this HLA class II allele (64). The presence of anti-NY-ESO-1 antibodies in patient sera also appeared to be correlated with expression of the HLA-DP β 1*0401 allele. Sixteen out of 17 melanoma patients who possessed significant anti-NY-ESO-1 antibody titers expressed HLA-DP β 1*0401, whereas only two out of eight patients with NY-ESO-1 positive tumors who did not have detectable titers of NY-ESO-1 antibodies expressed HLA-DP β 1*0401. These results provide support for the hypothesis that CD4+, HLA-DP β 1*0401-restricted T cells that recognized the NY-ESO-1₁₆₁₋₁₈₀ peptide may be involved with generating anti-NY-ESO-1 antibodies in patients who expressed this HLA class II allele.

4. CLINICAL APPLICATIONS

In general, current immunotherapies that have been developed for the treatment of patients with cancer have failed to have a significant impact on the survival of the majority of patients. Although it is not clear what is responsible for the lack of effective treatment in many of these patients, there are several potential mechanisms that may act to limit *in vivo* responses to immunotherapy. The outgrowth of tumors that have either lost or downregulated the expression of molecules involved with antigen recognition, including genes that encode the antigens and MHC molecules, as well as genes involved in antigen processing, may be responsible for some of these failures. Alterations in the expression of these products has been observed in certain tumors (66), and mutations or deletions of the β 2-microglobulin locus that result in an essentially complete loss of cell surface HLA class I expression have been observed in tumors derived from patients that have relapsed following immunotherapy (67). Nevertheless, these appear to generally represent isolated cases, and the analysis of cell lines as well as fresh tumor samples from melanoma patients has demonstrated that loss of HLA class I or tumor antigen expression is relatively rare. In addition, the ability to respond to immunotherapy does not appear to be correlated with the expression of either antigen or HLA class I gene products.

A number of new therapeutic options that are currently being evaluated may result in more effective treatment. Preliminary results of an ongoing clinical trial indicate that treatment of patients with a course of nonmyeloablative chemotherapy may enhance responses to adoptive immunotherapy carried out with tumor-reactive T cells (68). One of the major effects of the chemotherapy that was used in this trial may have been to significantly enhance the engraftment of tumor-reactive T cells, as tumor-reactive T-cell clones were found to persist at high levels in the blood of some of the patients in this trial following adoptive transfer. In a previous adoptive transfer trial carried out using highly reactive CD8+ tumor-reactive T-cell clones that did not employ this treatment, transferred T cells were not generally detected beyond 24 h following T-cell transfer, and clinical responses were not observed (69). The use of a variety of additional strategies that employ immunization with HLA class I- and class II-restricted epitopes, as well as the adoptive transfer of class II-restricted, tumor-reactive T cells may also result in enhanced therapeutic responses. Continuing studies will hopefully lead to a better understanding of the mechanisms involved with maintaining the survival of T cells with the appropriate specificity and activation phenotype *in vivo* and result in the eventual development of more effective immunotherapies for the treatment of patients with cancer.

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5

Targeting Viral Antigens for the Treatment of Malignancies

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1. INTRODUCTION

Viruses implicated in the development of human cancer include hepatitis B (HBV) and hepatitis C (HCV) viruses, human papilloma virus (HPV), Epstein-Barr virus (EBV), human T-cell lymphoma virus, and human herpes virus 8. Together they contribute significantly to the total incidence of cancer worldwide. Current work in each of these virus systems seeks to understand the mechanisms of viral action and identify strategies of immune intervention to combat viral infection and subsequent transformation. It is thought that oncogenic proliferation may be instigated by the presence and expression of viral oncogenes, which may be integrated into the host genome. Critical viral genes may also interfere with host genes, resulting in the activation of cellular proto-oncogenes and/or the inactivation of anti-oncogenes and their products. Targeting such viral proteins through various vaccination strategies offers both therapeutic and prophylactic strategies against viral induced malignancies.

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2. HISTORY

Views on the role of viruses in the development of cancer have been controversial during the major part of the last century. The belief that viruses have nothing to do with cancer was as widespread as the contention that most if not all tumors were virally induced. The debate began with the discovery by Peyton Rous in 1911 (1) that chicken sarcomas could be transmitted into healthy chickens with cell-free tumor filtrates. This infectious agent came to be called the Rous sarcoma virus. However, when rat and mouse tumor filtrates failed to induce tumors, enthusiasm subsided. It was thought that viruses might cause tumors in birds but not in mammals. In 1933, Richard Shope found that benign warts could be transmitted from the wild cottontail to the domestic rabbits by a papilloma virus (2). These initially benign rabbit papillomas could occasionally turn into carcinomas, suggesting a transition or tumor progression. Shope also showed that these virally induced warts that did not progress to carcinomas were rejected by an immunological host response, mediated by lymphocytes rather than antibodies. This was the first example of a tumor rejection response that targeted virally encoded proteins in transformed cells. Unfortunately, the implications of Shope's experiments were not appreciated at the time and so did not change the prevailing opinion that viruses do not cause human cancers.

In 1964 reports of the first human tumor virus, the EBV, were received with much skepticism. Even after the accumulation of abundant data on the biology and powerful lymphocyte-transforming abilities of EBV (3), and the persuasive evidence from massive World Health Organization studies, the reluctance to believe there could be a carcinogenic virus of man persisted. In contrast, the most recently found human tumor virus, Kaposi's sarcoma (KS) herpesvirus, was accepted as a tumor virus almost immediately, reflecting the dramatic change in opinions between 1964 and 1994. This change in perception of human tumor viruses was driven by the close associations of other viruses with cancer. The important role of other tumor viruses in the induction of certain cancers and leukemias have all contributed to this acknowledgement that virus can contribute to carcinogenesis. It is now evident that 20–25% of human cancer is virus associated and there are certainly other such agents that have yet to be identified (4). Of course none of the human tumor viruses are directly carcinogenic; rather each is necessary but not on its own sufficient to cause malignancy. In the absence of these infections, the cancers described here would be reduced by 95% with a significant reduction in morbidity and mortality, especially in developing countries. Mere infection, however, does not mean that cancer will result, since many more people are infected than will develop a malignancy. More likely, cancer can be seen as a rare outcome of a common infection (5). This proves to be one of the major problems in understanding the molecular causality of virally induced human cancers.

3. ONCOGENIC VIRUSES

The list of human viruses presently known to cause or contribute to tumor development comprise four DNA viruses, namely EBV, certain human papilloma virus subtypes, hepatitis B virus, and KS herpesvirus; and two RNA viruses, adult T-cell leukemia virus and HCV (Table 1). EBV is most directly involved in the causation of immunoblastomas that arise in immunodeficient persons, such as organ transplant recipients. EBV may also

Table 1
Viral Tumor Antigens

<i>Virus</i>	<i>Associated Malignancy</i>	<i>Target Antigen</i>
EBV Type I	Burkitt's lymphoma Gastric adenocarcinoma	EBNA-1, BARFO
EBV Type II	Nasopharyngeal carcinoma Peripheral T cell lymphoma Hodgkin's disease	EBNA-1, LMP1, LMP2, BARFO
EBV Type III	Post-transplant Lymphoproliferative disease	EBNAs 1,2, 3A, 3B, 3C LP, LMP1, LMP2, BARFO
HPV 16, 18	Anogenital carcinomas Head and neck carcinomas	E6, E7
HBV	Hepatocellular carcinoma	HBx protein, Pre/S2
HCV	Hepatocellular carcinoma	HCV core protein, E2
HHV-8	Kaposi's sarcoma, Castleman's disease, primary effusion lymphoma	kaposin, LANA, vFLIP, vCyc D
HTLV-1	Adult T-cell leukemia	Tax

play a role in Burkitt's lymphoma (BL), Hodgkin's disease, and nasopharyngeal cancer, as indicated by the regularity of its association with these tumors. Certain high-risk subtypes of human papilloma virus are known to cause cervical and anogenital cancers, as well as a portion of head and neck cancers. Human herpes virus 8 (HHV-8) is associated with Kaposi's sarcoma, Castelman's disease, and body cavity lymphoma. Hepatitis B and C contribute to the genesis of primary liver cancer. Furthermore, the human immunodeficiency virus (HIV) has been associated with the progression of many cancers due to its induction of a compromised immune system.

Malignant tumors express antigens that may stimulate the cellular and humoral immune system and serve as targets for antitumor immunity. Virally induced tumors usually contain integrated proviral genomes in their cellular genomes and often express viral genome-encoded proteins that may stimulate specific host immune responses. Therefore, independent of whether the viral infection is the oncogenic agent, it has been shown that viral antigens are expressed in the associated tumors and can be used as targets for preventive and therapeutic vaccinations. Cancers that do not naturally induce an immune response can still be susceptible to T-cell-mediated eradication when activated cytotoxic T lymphocytes (CTLs) are generated by vaccination. Therefore, the therapeutic approach to patients with preinvasive and invasive cancers is to develop vaccine strategies to induce specific CD8+ CTL responses to the relevant class I epitopes of viral proteins presented by tumors, thus stimulating the adaptive immune response to eliminate virally infected or transformed cells. CD4+ T cells are also critical in both effective viral and tumor CTL responses and lead to the destruction of the virus or tumor by CD8+ CTL. The role of CD4+ T cells in the priming phase of a tumor-specific CTL response is thought to be at the level of activating dendritic cells (DCs) to allow these cells to effectively activate naïve CD8+ T cells. There is now evidence for a broader role for CD4+ T cells in antitumor immunity in addition to providing help for CD8+ T cells. In fact, in the absence of CD8+ T cells, adoptive transfer of CD4+ T cells can lead to the elimination of tumor cells, suggesting a much larger role for CD4+ T cells than simply help for CTL (6).

This chapter will discuss those viruses where there is overwhelming evidence of a causal association between the virus and human malignancies and the potential to target viral antigens for cancer therapy.

3.1. EBV

The EBV is a human gamma herpesvirus with a tropism for B lymphocytes (7). It is considered the most highly transforming virus (5). Nevertheless, its interaction with humans is largely apathogenic. In fact, more than 95% of the adult population carries EBV as a lifelong asymptomatic infection (8). Tumor development induced by EBV can, therefore, be seen as an accident of immunosuppression or of cellular changes. Since its discovery in 1964, EBV has been associated with a number of human malignancies. These include BL, nasopharyngeal carcinoma, posttransplant lymphoproliferative disease (PTLD), and Hodgkin's disease. In highly endemic BL, EBV is present in 98% of cases. AIDS-associated BLs are EBV-positive in 30–40% of cases except for those that start in the brain, as they are 100% EBV-positive. Posttransplant immunoblastomas carry the virus in nearly 100% of cases, whereas the frequency of EBV carrying Hodgkin's lymphomas is about 50% (9).

Like other human herpesviruses, EBV causes latent or lytic infections. In latent infection, there are at least nine related proteins and two small nonpolyadenylated RNAs encoded by EBV DNA. These include EBNA-1, -2, -3A, -3B, -3C, BARF0, leader protein (LP), latent membrane protein (LMP)-1 and LMP-2, and EBV-encoded RNAs (EBER)-1 and -2. EBNA-1 is associated with cell proliferation, as is LMP-1. Functions of the other latent proteins and RNAs remain obscure, but are considered to be related to both cell proliferation and viral replication. There is now compelling evidence that this latent infection is controlled by a population of EBV-specific CTLs that recognize epitopes mainly derived from EBNA proteins (10). Therefore, a loss of CTL control can severely compromise the ability of the host to control the outgrowth of EBV-infected B cells. PTLD seen in transplant patients is a classic example where the loss of CTL control due to immunosuppressive therapy often results in the massive expansion of EBV-infected B cells (11). Knowledge on the role of CTL-mediated control of EBV-infected normal B cells allows us to utilize the adaptive immune system for the treatment of EBV-associated malignancies.

Three different types of latency phenotypes have been identified in EBV-associated malignancies, each of which gives rise to specific tumors originating from B cells or other cell types. BL represents a classic example of a latency I phenotype. Only the EBNA1 and BARF0 proteins are expressed in these transformed B cells. Although CTL responses to both of these antigens have been detected in the peripheral blood of healthy virus carriers, these CTLs are unable to recognize virus-infected cells expressing these antigens (12). This may be due to the fact that EBNA1 blocks its own processing for major histocompatibility complex (MHC) class I presentation, whereas BARF0 is not expressed at high enough levels to be recognized by specific CTLs (13). Furthermore, BL cells also express very low levels of antigen-processing genes (TAP-1 and TAP-2), which play a crucial role in the endogenous processing of CTL epitopes (14). Thus, BL cells resist CTL control by not only downregulating the expression of highly immunogenic antigens, but also blocking the endogenous presentation of their potential tumor-associated epitopes. In latency II EBV-associated malignancies like Hodgkin's lymphoma and nasopharyngeal carcinoma, four specific EBV genes, EBNA1, BARF0, LMP1, and LMP2, are main-

tained. As opposed to EBNA1 and BARF0, both LMP1 and LMP2 are moderately strong targets for virus-specific CTLs. Several epitopes that are restricted through common human leukocyte antigen (HLA) alleles have been identified within these two antigens (15,16). As exemplified by lymphoproliferative disease, the latency III phenotype is characterized by the expression of the nine EBV gene products: six EBNA s and three LMPs. Because tumor cells from these malignancies are highly immunogenic, latency III malignancies are seen only in those individuals who are severely immunocompromised by solid-organ or stem-cell transplantation, congenital immunodeficiency, or HIV. Knowledge of these phenotypic differences in EBV-associated malignancies provides an important basis for the development of immunotherapeutic strategies against viral antigens.

3.1.1. IMMUNOTHERAPY AGAINST EBV ANTIGENS

The most promising vaccination strategies for the immunotherapy of EBV-associated malignancies seek to elicit or boost the EBV-specific cellular immune response against EBV latency proteins. Unfortunately, the low expression of immunogenic antigens and loss of antigen-processing function in type I malignancies severely restricts the potential for immunotherapeutic strategies against viral antigens. However, recent studies have provided promising alternatives. It has been shown that the downregulation of MHC class I molecules in BL cells can be reversed by CD40 crosslinking *in vitro* (17). Despite the significant upregulation of MHC class I molecules and TAP-1 and TAP-2, the expression of EBV-associated proteins was not increased, indicating that CTL therapy for BL may be problematic. However, the upregulation of MHC class I molecules in BL cells may reveal other lymphoma-specific antigens to target for immunotherapy. Another promising strategy utilizes the finding that CD4-positive EBNA-1-specific CTLs can efficiently recognize EBV-infected B cells (18). Since BL can efficiently process class II-restricted epitopes, these CD4 CTLs may recognize EBV-positive BL cells and would provide an ideal tool for adoptive immunotherapy.

As mentioned earlier, LMP1 and LMP2 antigens expressed in type II malignancies can be used as targets for CTL immunotherapy. Adoptive transfer of polyclonal EBV-specific CTLs into patients with advanced Hodgkin's disease have shown improvement, including resolution of some symptoms and stabilization of the disease (19). However, adoptively transferred CTLs have not been capable of promoting complete disease regression. Another promising approach for type II malignancies involves the stimulation of CTLs with autologous dendritic cells (DCs) transduced with viral vectors that express the LMP antigens (20). This DC-based stimulation leads to a preferential expansion of LMP-specific CTLs and may be more effective for immunotherapy. Finally, a DNA vaccine based on LMP epitopes may hold the most promise for efficient treatment of type II malignancies. This approach would eliminate the challenges, both cost and technical, of adoptive T-cell therapy and by targeting relevant epitopes would avoid the use of highly oncogenic forms of full-length LMP.

The expression of all EBV latent antigens should render the type III malignancies ideal targets for T-cell-based immunotherapy. However, due to severe immunodeficiency, mortality from PTLD can be as high as 50% (21). Adoptive transfer of EBV-specific CTLs from the bone marrow donor has been successfully used to resolve PTLD in the bone marrow recipient (22). Unfortunately, the major challenge arises in the case of solid-organ transplantation where adoptive immunotherapy is unlikely to be successful due to high levels of immunosuppressive drugs and could present a significant risk to the

engrafted organ. Thus, immunotherapy with infusion of ex vivo-activated specific CTLs seems to be more promising. EBV-specific CTLs used in these settings would likely have to be derived from autologous T lymphocytes, which may be technically difficult due to the underlying immunosuppressed state. However, adoptive transfer strategies based on CTL epitopes from EBV latent antigens are currently under development and could overcome the difficulty of isolating and propagating tumor-specific T cells from advanced cancer patients. It has been shown that the retroviral transfer of genes encoding tumor-specific T-cell receptors (TCRs) into peripheral blood T cells could enhance antigen-specific immunity by increasing the frequency of tumor-specific T cells (23). This approach, which uses the ability to transfect a patient's T cells with a TCR specific for a tumor antigen in vitro, may, after readministration of the T cells to the patient, be effective at targeting tumor antigen-expressing cells. Consequently, TCR transfer technology offers the potential to generate a bank of TCR genes with specificity to various viral tumor antigens that may be selected for any patient with tumor cells expressing the appropriate antigens. Although it is difficult to imagine a single therapeutic strategy for all EBV-associated malignancies, any strategy that can alter the stability of the critical EBNA1 antigen in vivo will ultimately play a crucial role in the future of EBV therapy.

3.2. Viral Hepatitis

It is well established that chronic infection by HBV and HCV viruses are major risk factors for the development of hepatocellular carcinoma (HCC). HBV is considered the dominant cause of HCC in developing countries of sub-Saharan Africa and Asia, whereas HCV is the major risk factor in developed countries (24). The World Health Organization has stated that liver cancer is the most common cancer in the world. About half of the cases are due to chronic HCV infection, with most of the remainder being due to HBV infection (25). Recently in Japan, it has been shown that over 95% of patients with HCC are infected with HBV or HCV, or both (26,27). Synergistic effects leading to the carcinogenicity of these two viruses have been shown in many populations, as well as between viral infections and other environmental factors, such as alcohol or chemical carcinogens. For these reasons it has been proposed that the hepatitis viruses have only an indirect involvement in HCC via hepatic inflammation. Therefore, although overwhelming epidemiological relationships have clearly been established, the direct role these infections play to cause HCC has remained controversial and largely unknown. Recently studies using transgenic mice have shown that the product of the *HBV X* gene (HBx protein) and the core protein of HCV have oncogenic potential (28,29), thus indicating that HBV and HCV are directly involved in hepatocarcinogenesis. It is proposed that these viral proteins contribute to the development of HCC at such a high incidence by accelerating the multi-stages of hepatocarcinogenesis. In other words, the overall effects achieved by the expression of HCV core protein and HBx would be the induction of HCC, even in the absence of a complete set of genetic aberrations normally required for carcinogenesis.

3.2.1. HBV

It is estimated that roughly one-third of the world population has been infected with HBV and there are about 350 million (5–6%) persistent carriers (30). HBV causes 80% of all liver cancer of human beings and approx 2 million people die every year as a consequence of infection with this virus. The first direct evidence of an association between chronic HBV infection and HCC was a report of five patients with HCC whose

serum was positive for HBV surface antigen, HBsAg (31). Since then it has been shown that the prevalence of HBsAg in patients with HCC is appreciably higher than it is in patients with cirrhosis or chronic hepatitis, providing further support for a specific carcinogenic effect of the virus. Examination of the viral DNA sequences present in HCC tumor cells has shown that in a large proportion of them, sequences encoding for HBV HBx viral proteins are integrated. This finding has prompted intense investigation of the role of this protein in hepatocarcinogenesis. The seminal observation that HBx transactivates cellular genes controlling cell growth suggested that HBx might participate in the transformation of hepatocytes by HBV. Consistent with this, it has been shown that HBx can induce cell proliferation in quiescent fibroblast (32), and also deregulate cell-cycle check points (33). Furthermore, HBx can induce HCC in transgenic mice (34,35). Truncated forms of the Pre/S2 envelope protein, generated from integrated viral genomes, may also be involved in the liver cell transformation (36). It has been reported that up to 25% of HCCs may contain such truncated sequences.

Effective vaccines for the prevention of HBV infection were first introduced in 1981, using inactivated human HBsAg positive serum. The HBsAg particles were subsequently purified from seropositive human sera and used in the plasma-derived vaccine. Advances in genetic engineering enable the large-scale production of HBsAg protein product by viral expression vectors. The effectiveness of HBV vaccination in the primary prevention of chronic HBV infection and HCC has already been demonstrated in pilot vaccination projects. In Taiwan, universal HBV vaccination was initiated with newborns of HBsAg-positive mothers (37,38). The average annual incidence of HCC in children has significantly decreased since then. However, economical and logistical reasons make implementation of HBV immunization programs problematic in many of the endemic areas. Efficient delivery of vaccination thus remains an important priority that will define the objective of eliminating HBV-related HCC.

3.2.2. HCV

In 1989, with the advent of a serum assay for antibodies to HCV (anti-HCV) it became possible to measure the relevance of HCV in the development of HCC. There are 200–400 million chronic HCV carriers in the world and about 20% will go on to develop HCC. Nucleotide sequences from HCV and virus-specific protein expression have been demonstrated in HCC tissue (39). However, the molecular basis of HCV-associated liver carcinogenesis is poorly understood and a variety of normal and abnormal HCV-encoded proteins have been implicated. The nuclear localization of the core protein has been repeatedly demonstrated in patients and its function questioned (40). The core protein is the fundamental unit for encapsidation of the genomic viral RNA during the process of viral maturation that takes place in the cell cytoplasm, not the nucleus, attracting much interest. The finding that the core protein may have transregulatory function on cellular and viral promoters suggests that HCV core protein may be directly involved in HCC. Other data suggest that HCV core protein may also have an important role in the promotion of cell growth by downregulating p53, thus weakening cellular tumor suppressor functions (41). Finally, as with the HBx protein, HCC developed in mice transgenic for the HCV core gene. Therefore, the core protein offers a promising viral antigenic target for HCV-related HCC therapy. Therapeutic immunization for HCV is even more important than in HBV infection because of the lack of an effective vaccine against HCV infection.

3.2.3. IMMUNOTHERAPY AGAINST HBV AND HCV ANTIGENS

Genetic vaccination represents a real alternative for the prevention and treatment of HBV and HCV viral infections. This immunological approach is based on the stimulation of broad cellular immune responses to viral proteins by using nucleic acids that express viral gene products. In vivo expression of the immunogen mimics that observed during the viral infection and leads to a natural presentation of antigens. This strategy, seen primarily as preventive, also holds great potential for therapy considering the pivotal role played by the immune response in the resolution of acute and chronic HBV and HCV infection. It is presently believed that the failure to eliminate the tumor by the immune system does not result from the absence of recognizable HCC tumor antigens but rather from the disability of these antigens to stimulate an effective immune response. Therefore, vaccination that induces potent CTL responses may offer great potential for eliminating HCCs. A number of recent advances suggest that development of therapeutic vaccines for HBV and HCV, and prophylactic vaccines for HCV are now possible. Humoral and cellular T helper and CTL responses have been identified in mice using plasmid DNA constructs expressing HBsAg and HBV core antigen (HBcAg), as well as HBx (38–40). Plasmid DNA constructs expressing different structural and nonstructural genes of HCV have also been tried, with the core gene being most frequently used. Envelope genes E1 and E2 of HCV and Pre/S2 of HBV are also possible candidates for immunization (42,43). Future definition of the role that viral proteins play in the transformation of liver cells should lead to an understanding of the mechanism of HCC that is associated with HBV and HCV infections and should be important in the development of immunotherapeutic strategies.

3.3. *Human Papillomavirus*

Carcinomas of the anogenital tract, particularly cancer of the cervix, account for almost 12% of all cancers in women, and so represent the third most frequent gynecological malignancy in the world (44). It is well established that chronic infection of cervical epithelium by human papilloma viruses (HPVs) is necessary for the development of cervical cancer. HPV DNA has been demonstrated in more than 99% of all tumor biopsy specimens, with high-risk HPV16 and HPV18 being most prevalent (45). HPVs are double-stranded DNA viruses that infect basal and suprabasal layers of stratified epithelium. The early genes, which include E1, E2, E4, E5, E6, and E7, code for proteins involved in viral DNA replication, transcriptional control, and cellular transformation. Late genes encode the major viral capsid protein, L1, and a minor capsid protein, L2. It has been demonstrated that E6 and E7 expression is required for the immortalization of primary cells as well as for maintenance of the transformed state (46,47). E6 and E7 expression delay keratinocyte differentiation and stimulate cell cycle progression, allowing the virus to utilize host DNA polymerases to replicate its genome. Specifically, the E6 protein has been shown to bind to p53 and target its degradation by the proteasome (48). p53 is a central transcription activator that regulates responses to stress and DNA damage. Loss of p53 leads to genetic instability and rapid malignant progression. The other critical oncogene, E7 protein, binds to the retinoblastoma protein (pRb) (49). This interaction occurs primarily with the hypophosphorylated form of pRb causing the release of active E2F transcription factors, which in turn stimulate expression of genes involved in cell cycle progression and DNA synthesis. The E6 and E7 viral genes are

expressed at low levels in proliferating basal cells, but are highly expressed in HPV-associated genital cancers (50,51). As a consequence, the E6 and E7 proteins can serve as major targets for the cell-mediated immune response and are, therefore, attractive targets for specific immunotherapies.

3.3.1. HPV IMMUNOTHERAPY

Cell-mediated immune responses are believed to be essential in controlling HPV infections as shown by the increased frequency of HPV-associated tumors in individuals treated with immunosuppressive drugs or suffering from AIDS (52,53). Thus, vaccination strategies have been developed to enhance the cellular immune responses against E6 and/or E7 viral proteins and eliminate HPV-infected cells. The goal of therapeutic immunization against HPV-induced carcinomas is to induce cellular components of the immune system to recognize and attack cells infected with HPV, including malignant tissue. Modulation of the immune response to HPV-infected cells is possible through many different vaccination strategies. Many HPV vaccine strategies have successfully elicited immune responses against HPV16 E6 and E7 epitopes and have prevented tumor growth upon challenge with HPV16-positive tumor cells in mice. The vaccination strategies have included viral vectors (54,55), synthetic peptides (56), recombinant proteins (57–59), chimeric virus-like particles (CVLPs) (60–62), and plasmid DNA (64,64). Unfortunately, DNA vaccination with intact E6 or E7 genes has a risk of oncogenic transformation through integration of recombinant genes into the host genome whereas peptide vaccines are limited to predetermined target HLA alleles and could possibly induce T-cell tolerance (65). Potential drawbacks from the use of CVLPs or viral vector systems include the induction of neutralizing antibodies against viral structural proteins in preimmune patients or after subsequent vaccination (66). The use of an alphavirus delivery vector may eliminate most of these drawbacks. Alphaviruses such as Venezuelan Equine Encephalitis (VEE) virus replicate the RNA of interest in the cell cytosol and are cytopathic, thereby significantly reducing the risk of integration of E6 and E7 into the cellular genome. By infecting DCs, VEE replicon particles (VRPs) target expression to lymphoid tissue, a preferred site for the induction of immunity (67,68). Furthermore, repeated immunizations may be possible since there is no widespread existing immunity to VEE in humans and effective vaccination can be obtained with very low doses of VRPs. Another promising vaccination strategy is a multiepitope plasmid DNA vaccine encoding several CTL epitopes from multiple HLA types, rather than entire proteins (63). This strategy eliminates the possible risk of oncogenic transformation and the downfall of targeting one HLA restriction element. Thus far, clinical trials have not been very successful in achieving significant tumor regression, most likely because the diseases were too advanced to treat with immunological intervention (69,70). However, their ability to stimulate HPV-specific immune responses and overcome immunosuppression in patients is very promising (71). Further studies that are directed toward patients with early cervical lesions should result in stronger cell-mediated immune responses and improved clinical outcomes.

3.4. HHV-8

HHV-8 was first detected in KS tissues from an AIDS patient in 1994 (72). Since its initial discovery, HHV-8 has been found in all forms of KS, as well as in primary effusion B-cell lymphomas and Castelman's disease, also known as giant lymph node hyperplasia. KS is at least 20,000 times more common in HIV-infected patients as compared to

the general population and represents the most common AIDS-associated cancer (73). Among HIV-negative patients, KS is primarily a disease of the elderly, which is consistent with nonspecific age-related decline in immune surveillance among those persons infected with HHV-8. Much like EBV, HHV-8 infects dividing B cells. It is suggested that reactivation of HHV-8 and expansion of the HHV-8 latently infected cells may be required for KS development. For other herpesviruses, lytic infection is controlled by the immune system, whereas a decrease in immune surveillance is associated with virus reactivation. Due to its prevalence in HIV-1 immunosuppressed patients, this is likely to be the case with HHV-8 as well. Although there is compelling epidemiological evidence that HHV-8 is involved in KS pathogenesis, the molecular mechanisms are largely unknown.

HHV-8-associated malignancies such as KS have been found to express a unique protein called kaposin. The kaposin gene induces tumorigenic transformation in transfected cells and is considered to be necessary to maintain the transformed state (74). Latency genes v-cyc D, v-FLIP, and LANA may also be involved in cancer progression because of their capability of promoting cell growth by direct or antiapoptotic effects. In fact, although KS cells appear to express these gene in early stages, their expression increases with lesion stage (75–77). This suggests that the continuous expression of these genes may be required for KS progression to a true cancer. Both v-cyc D and LANA are likely to play a role in KS progression because of their effects on cell growth. Like the HPV E7 protein, these viral proteins mediate the phosphorylation of Rb thereby inducing cell cycle progression (78). LANA is one of the few viral proteins ubiquitously expressed in cells latently infected with HHV-8 and is often used as a serological marker. v-FLIP is thought to interfere with KS cell apoptosis signaled by the tumor necrosis factor (TNF) family of receptors (79). This function may be critical for preventing CTL-induced apoptosis, which might be otherwise induced as a result of the viral infection. Together, data support the concept that HHV-8 latency genes may play a key role in the progression of KS by providing KS cells with growth and/or antiapoptotic signals and may be potential targets for therapy. Furthermore, the HIV-1 Tat protein is a transcriptional activator that appears to be responsible for the higher incidence of KS in HIV-1-infected people (80). In this respect, Tat acts as a progression factor for AIDS-KS and therapy against HIV-1 may be effective against KS as well.

3.4.1. HHV-8 IMMUNOTHERAPY

Targeted cancer therapies in AIDS patients present an additional challenge in the field of tumor immunotherapy. With the majority of HHV-8-related cancers afflicting severely immunocompromised patients, new strategies to induce specific cellular immunity are required. Although several chemotherapeutic agents have proven effective in controlling KS, the growing understanding of its pathogenesis provides a strong rationale for the development of immunotherapeutic strategies against HHV-8 latent proteins. Targeting HHV-8 by interfering with the stability of LANA is an approach that is being widely investigated. Although a complete understanding of the pathogenesis of KS is required to establish a pathogenetic therapy for KS, different factors including HHV-8 latent proteins, oncogenes, and tumor suppressor genes as well as HIV-1 Tat should be investigated to identify therapeutic agents against the early stages of KS or the nodular tumor stage of the disease.

3.5. HTLV

The human T-cell leukemia virus-1 (HTLV-1) is a retrovirus that causes adult T-cell leukemia (ATL), a malignant disorder of mature CD4+ cells. It is estimated that 10–20 million people worldwide are infected with HTLV-1, which is endemic to southern Japan, Africa, and the eastern parts of South America. ATL occurs in 1–2% of infected carriers generally 20–30 yr after infection (81). Epidemiological studies have shown that ATL develops mainly among individuals that were infected in infancy, primarily through breast milk. This long latency period of ATL suggests that the accumulation of genetic mutations, in addition to HTLV-1 infection, may be required for the induction of ATL. In contrast to other tumor viruses, HTLV-1 does not contain a classical oncogene and the integration of the virus genome in leukemia cells is at random (82,83). Therefore, the mechanism of HTLV-1-induced oncogenesis has not been identified. Much attention has focused on the oncogenic potential of the HTLV-1-encoded Tax protein. Tax is required for replication of the virus. It is also the chief target antigen recognized by the strong CTL response against the virus. As well as activating the transcription of HTLV-1 genes, Tax has been shown to stimulate the expression of various growth-related cellular genes, including oncogenes. Furthermore, Tax has been shown to play an important role in the induction of tumors in transgenic mice (84). These findings strongly suggest a role for Tax in pathogenesis of ATL.

3.5.1. IMMUNOTHERAPY AGAINST HTLV-1

Recent research on the molecular virology and immunology of HTLV-1 shows the importance of the host's immune response in reducing the risk of ATL, and is beginning to explain why some HTLV-1-infected people develop serious illness whereas most remain healthy lifelong carriers. There is neither a vaccine against the virus, nor a satisfactory treatment for the malignancy. However, vaccination strategies may be successful at inducing a strong immune response against HTLV-1, most notably a CTL response directed against the immunodominant epitopes of Tax. Anti-Tax CTLs are unusual in their extreme abundance: Up to 10% of circulating CD8+ T cells can recognize just one epitope of the HTLV-1 virus. The strength of this anti-HTLV-1 CTL response might explain why some HTLV-1-infected people develop ATL, whereas others effectively suppress the replication of HTLV-1 and remain healthy. Therefore, therapy against HTLV-1 and subsequent ATL should focus upon upregulating this potent CTL response against the immunodominant epitope of the Tax protein.

4. CONCLUSION

Over the past several decades, there has been enormous advance in our understanding of the pathogenesis and immune control of virally associated malignancies. It is generally accepted that tumor formation by a virus is likely to be an accidental event. Most tumor viruses are not tumorigenic in their natural hosts under normal circumstances and there is no apparent evolutionary advantage in causing a tumor since it does not enhance lytic progeny and is deleterious to the host. Immunologic surveillance is understood to play an important role in preventing virus induced tumorigenesis. As seen with many of these viruses, a normally benign viral infection in persons with intact immunity will induce a wide range of carcinomas in the setting of transplant-related or HIV-induced immunosuppression. Thus, a large number of healthy persons can be infected and not

manifest tumor development unless they are confronted by severe immunosuppression or an accumulation of oncogenic mutations. Understanding the relationship between viruses and human cancer is critical for the development of effective prophylactic and therapeutic strategies. By acknowledging this important health risk, it may be possible (a) to educate people on how to avoid infection, (b) to develop vaccines to prevent viral transmission, and (c) to target viral antigens for immunotherapy. This chapter has presented possible viral antigens that may be promising at inducing antiviral immune responses and tumor regression. The spectacular success of preventing viral disease, such as hepatitis B, by the generation of neutralizing antibodies offers hope that immunoprophylaxis against relevant viral antigens could lead to a major reduction in the incidence of cancer. Neutralizing antibodies may be an effective way of preventing viral infection and spread, but cell-mediated immune responses will ultimately be required for the resolution of established disease. Although therapy for each virus/cancer relationship will be different, the common goal remains to elicit strong CTL responses against viral proteins. One of the most promising strategies may be to adoptively transfer viral antigen-specific TCRs to a patient's T cells. This approach offers the ability to target a wide range of cancer types by tumor-specific TCRs and may overcome the immunosuppression seen in many patients. Furthermore, the ongoing characterization of tumor antigens, which may result in additional tumor-specific TCRs, makes this approach more universal and could become an important tool in the treatment of cancer.

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6

Immune Defects in Cancer

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REFERENCES

1. INTRODUCTION

The host immune system is one of the most important elements of antitumor defense. The idea of cancer immunosurveillance proposed by Sir Macfarlaine Burnet and Lewis Thomas (1,2) has been recently highlighted in experiments with gene knockout mice. Compared with wild-type mice, mice lacking sensitivity to either interferon- γ (IFN- γ receptor-deficient mice) or all interferon (IFN) family members (i.e., Stat1-deficient mice) developed tumors more rapidly and with greater frequency when challenged with different doses of the chemical carcinogen methylcholanthrene (3). Mice lacking lymphocytes and IFN- γ ($RAG2^{-/-} \times STAT1^{-/-}$ mice) also had an increased incidence of spontaneous mammary tumors (4). The development of tumors and metastatic disease in nonimmunocompromised hosts can be considered as a result of an inadequate immune response against tumors and the evasion of tumor cells from the host immune system (5). The correlation between the presence of tumor-infiltrating lymphocytes and survival of patients with melanoma (6), breast (7), bladder (8), colon (9), neuroblastoma (10), ovary (11), and prostate (12) cancers gives hope that the manipulation of tumor escape mechanisms, by immunotherapeutic interventions, may provide clinical benefits. In tumor-bearing animals and cancer patients, however, the immune response against tumors is markedly depressed. The fact that the resection of progressively growing solid tumors restores protective antitumor immunity in experimental models of cancer (13) demonstrates the ability of the immune system to defend against an established tumor. Although the relationships between tumor cells and the host immune system are still poorly understood, two groups of tumor escape mechanisms can be described.

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The first group includes mechanisms based on the tumor cell nature. They involve the downregulation of major histocompatibility complex (MHC) class I and the transporter associated with antigen-processing (TAP) genes (14) as well as costimulatory molecules (15). This results in a failure to present tumor-associated antigens to T lymphocytes, the main antigen-specific cells mediating strong antitumor defense (14). Another group of mechanisms is associated with the function of the host immune system and their ability to recognize and eliminate tumor cells. In this chapter we will focus on these mechanisms of tumor escape.

Different immunocompetent cells are responsible for tumor cell killing. These cells include: (a) antigen-specific cytotoxic T lymphocytes (CTL, or Tc cells) and Tc precursors that produce IFN- γ ; (b) T helper (Th) cells, which produce interleukin-2 (IL-2) and IFN- γ ; (c) NK/NKT (natural killer and natural killer T) cells; and (d) antigen-presenting cells (APCs), such as dendritic cells (DCs), macrophages, monocytes, and B cells. Only T cells can directly recognize tumor cells via an antigen-specific mechanism. CTLs detect target tumor cells by recognizing 8–10 mer antigenic peptide fragments of endogenous proteins, which are presented by MHC class I molecules on the tumor cell surface. Th cells recognize 12–20 mer antigenic peptides that fit the MHC class II groove on the surface of APCs and modify the activity of CTLs by the production of regulatory cytokines. NK/NKT cells are involved in the nonspecific innate antitumor immune responses. APCs are responsible for the presentation of antigens and the delivery of costimulatory signals to Th and Tc cells. APCs, together with antigen-activated Th cells, provide the cytokine support for antigen-specific CTL clonal outgrowth. The dysfunction of any component of the immune system might have an impact on tumor progression.

2. T-CELL DYSFUNCTION IN CANCER

One group of mechanisms affecting tumor escape involves the induction of T-cell defects. They include nonspecific suppression of T-cell function, antigen-specific nonresponsiveness (anergy/tolerance), immune deviation, and central or peripheral deletion of T cells by apoptosis. Alone or in combination, these mechanisms may be responsible for a lack of tumor rejection.

2.1. *Systemic and Local Suppression of T Cells*

Immunosuppressive mechanisms that promote tumor growth include suppressive factors directly produced by tumor cells and those that are produced by other cells, although induced by the tumor. The most studied suppressive factor that can be produced by both the tumor and immunocompetent cells is the transforming growth factor- β (TGF- β). TGF- β inhibits differentiation of both CD4+ and CD8+ naïve T cells via different transcriptional activators (16). Blocking of TGF- β signals in T cells in mice leads to the generation of potent CTL responses that results in tumor rejection (16). In addition to its direct effect on T-cell function, TGF- β also inhibits T cells indirectly through its effects on APCs (16). An increased concentration of TGF- β in serum of pancreatic cancer patients has been positively correlated with a loss of CD3 ζ -chain in tumor-infiltrating lymphocytes (17). Other suppressive factors that impare T-cell function systemically (with an impact on proliferative responses to mitogens and cytokine production) include PGE2 (18), prostate-specific antigen (PSA) (19), and colon cancer mucin (20). These

factors might significantly impare tumor-infiltrating lymphocyte function because of the expected increased concentrations of these factors in the tumor microenvironment.

Local immunosuppression could also be associated with the overexpression of metalloproteases (MMP)-1, MMP-2, and MMP-9 in cervical cancer tissues. This resulted in an intratumoral release of a cleaved IL-2 receptor (IL-2Ra) from the surface of tumor-infiltrating lymphocytes (21). Because of the critical role of IL-2Ra in the maintenance and expansion of CTLs in the tumor site, rapid cleavage of IL-2Ra by MMP-9 was suggested to be responsible for the downregulation of the proliferative capability of cancer-encountered T cells (21).

The indirect influence of tumors on the development of a systemic immunosuppression can be attributed to hyperproduction of IL-10, oxygen metabolism intermediates, and some enzymes. IL-10 is a type 2 cytokine that is produced by APCs and Th2 cells. It is involved in the development of T-cell anergy, promotion of Th2 responses, and inhibition of Th1 responses, which are important for the generation of efficient antitumor responses. Increased production of oxygen metabolites by macrophages isolated from the metastatic lymph nodes of patients with malignant melanoma was found to be responsible for decreased CD3-mediated stimulation of T cells and the reduction of CTL and NK cell activity (22). The inhibitory effect of macrophages on melanoma-specific CTL lines and NK cells was abrogated in the presence of catalase, a scavenger for H₂O₂. The mechanism of H₂O₂-induced immunosuppression by monocytes/macrophages derived from the blood of cancer patients was attributed to the inhibition of Th1 cytokine production in memory/activated T cells, which correlated with the blocking of NF-κB activation (23) and reduction of phosphorylation and activation of JAK3/STAT5 signal transduction proteins (24). Increased oxidative stress in cancer patients has been suggested as one of the mechanisms behind the tumor-induced immunosuppression, and dietary supplementation of antioxidative formulation that included vitamin E, vitamin C, and selenium significantly increased number of CD3-stimulated IFN-γ-producing CD8+ CD45RO+ memory T cells in patients with advanced colorectal cancer (25). The increase in the number of phorbolmiristylacetate-ionomycin-activated T cells producing IL-2 following antioxidant therapy was statistically significant for all subsets of T cells (CD4+, CD8+, CD45RO-, CD45RO+) (25). Further, the short-course (for 2 wk) antioxidant therapy resulted in increased CD4:CD8 T-cell ratios. Whether the similar treatment would have an impact on the efficiency of vaccination against cancer, and whether other cancers would show similar results of antioxidative treatment remain to be elucidated.

Another mechanism that mediates both the direct and indirect immunosuppressive capability of tumor includes the overexpression of immunosuppressive, IFN-γ-inducible enzyme, indolamine 2,3-dioxygenase (IDO), by mononuclear cells evading tumors and tumor-draining lymph nodes, and by tumors itself (26,27). This enzyme is responsible for the catabolism of tryptophan (the amino acid that is critical for T-cell function), and a competitive inhibitor of IDO, 1-methyl-tryptophan (1-MT), can prevent the interference of APCs with T-cell function (27,28). Recent studies demonstrated that murine IDO-negative Lewis lung carcinoma recruited IDO-positive mononuclear cells in the tumor site and draining lymph nodes. Sustained delivery of 1-MT inhibited IDO and significantly delayed carcinoma cell growth in syngeneic mice and improved T-cell activity in the presence of carcinoma cells (29). These data provide the first evidence that IDO can contribute to tumor-induced immunosuppression. Interestingly, human monocyte-

derived DCs upon activation with CD40 ligand and IFN- γ or in the presence of activated T cells produce IDO that results in the inhibition of T-cell proliferation (28). Whether the overexpression of IDO in some tumors has an impact on T-cell function, and whether macrophages/monocytes and/or DCs in cancer patients overexpress IDO in the tumor site and significantly contribute to the suppression of tumor infiltrating lymphocyte function, are critical issues for future studies.

2.2. T-Cell Anergy/Tolerance

2.2.1. T-CELL ANERGY IN CANCER PATIENTS

Selective T-cell nonresponsiveness to a particular antigen, as shown by defective antigen-specific IFN- γ production and proliferation, has been repeatedly reported using murine tumor models. T-cell anergy has been shown to be an early event in tumor progression (30). Naïve CD4+ T cells specific for a tumor-associated antigen, transferred into tumor-bearing mice, had a significantly diminished response to the peptide antigen (30). In clinical settings, the circulating CD8+ T cells, specific for melanoma-associated antigens MART-1 or tyrosinase, were found to be unable to lyse melanoma target cells or produce cytokines. The ability of these cells to lyse Epstein-Barr virus-pulsed target cells or generate allogeneic responses was not impaired (31), indicating the specificity of anergy. Stimulation of T cells from melanoma patients with melanoma peptides did not induce the upregulation of CD69 expression, indicating the T-cell functional defects (31). Vaccination of melanoma patients increased frequencies of melanoma antigen-specific CD8+ T cells identified by human leukocyte antigen (HLA)/peptide tetramers, however, no tumor regression was determined (32). Other studies demonstrated that vaccination-induced T cells with an increased level of IFN- γ mRNA, which, however, was not accompanied by a significant inflammatory response or CD4+/CD8+ T-cell accumulation (33,34). Detection of these antigen-reactive T cells in the tumor did not correlate with clinical response. This demonstrates that although tumor-specific T cells can be detected in blood or in human tumors following vaccination against tumor antigens, it is not clear whether these cells are functional, anergic, or undergoing apoptosis.

2.2.2. SIGNALING DEFECTS IN T CELLS

Classical anergy might be induced when the T-cell receptor is engaged by the antigen in the absence of G1 to S phase cell cycle progression (35–37). Exogenous or locally secreted IL-2, however, may overcome anergy (35). Absence or a dramatic deficit of IL-2 production is considered one of the main mechanisms of inducing T-cell anergy when the antigen receptor is engaged. The anergic T cells display a proliferation defect and impaired production of IL-2 and alterations in T-cell signaling. Deficient activation of lck, ZAP-70, Ras, ERK, AP-1, JNK, and NFAT molecules was reported (reviewed in ref. 38). The anergizing stimuli activate the protein tyrosine kinase fyn, increase intracellular calcium levels, and activate RAP1. There is upregulation of the cyclin-dependent kinase inhibitor p27kip1 that may block cell cycle progression (38). Other data, however, do not confirm the correlation of p27kip1 levels and anergic phenotype (35). Whether T cells that are unresponsive in cancer patients possess functional and signaling profiles similar to anergic T cells has not been well characterized. This is partly due to the difficulty of assessing signaling events in tumor-specific T cells because of their low numbers. Few studies reported the serial cytokine gene expression analysis within the tumor environment in melanoma patients (33). Quantitative RT-PCR performed from RNA isolated

directly from the aspirated material obtained from the same lesion before and after vaccination showed a less than twofold increase in IFN- γ mRNA expression post-therapy (normalized to CD8 expression) (33). The same samples, however, showed no increase in IL-2 mRNA expression and no tumor regression was determined. This demonstrates that the vaccine induced increased the local T-cell reaction as assessed by determining intratumoral T-cell cytokine expression, is not sufficient to eradicate the tumor. Presumably, the defective signaling or locally generated immunosuppressive environment may affect the expression of IL-2 in CTL or in CD4+ Th cells resulting in anergy.

Systemic and local immunosuppression in animal tumor models and cancer patients is associated with a hyporesponsiveness to challenge with common recall antigens, diminished T-cell function (most significant in tumor-infiltrating lymphocytes), and reduced cytotoxic effector function (39,40). These alterations are linked to abnormal expression and/or function of signaling events that involve T-cell receptor (TCR). The most studied defect in T-cell signaling is the reduction in expression of the TCR complex ζ chain, which is a part of the TCR-signaling pathway. Signaling that involves the ζ chain is necessary for T-cell activation and effector function (41). T cells from patients with melanoma, ovarian cancer, cervical cancer, and Hodgkin's disease display a reduced expression of the ζ chain. Reduction in ζ chain expression has been correlated in some studies with a decrease in either proliferation or Th1 cytokine production and disease progression (40,42).

The factors that contribute to the reduction in ζ chain levels in cancer patients include overproduction of hydrogen peroxide by activated granulocytes (blood) and macrophages (tumor) (22,43), activation of caspases, and repeated stimulation via the TCR or by tumor necrosis factor (TNF) (41,44). Studies in animal tumor models and renal cancer patients have demonstrated defective activation of the transcription factor NF- κ B in tumor-infiltrating lymphocytes and peripheral blood-derived T cells (42,45,46). This defect is noted in T cells from 50% of renal cancer patients and in less than 5% of patients with no evidence of disease after treatment or malignant disease-free individuals (46). NF- κ B is a cell survival factor, which regulates the expression of various antiapoptotic genes (47). There is evidence for a preferential role of NF- κ B in Th1- but not Th2-cell responses (48). Whether the NF- κ B defect in patients' T cells is related to increased sensitivity to apoptosis and/or to a bias for a Th2 cell response needs to be assessed. Inhibition of NF- κ B activation may be partly due to soluble products present in the tumor microenvironment. Inhibition of NF- κ B by hydrogen peroxide occurs primarily in memory T cells and correlates with the reduced expression of Th1 cytokines (23). Immunosuppressive gangliosides present in supernatants from renal cancer patients also suppress NF- κ B activation in T cells (49). Inhibition of antitumor CTL activity of established T-cell lines correlates with suppression of NF- κ B activity. Finally, in a transgenic animal model, the induction of tolerance to self-antigens coincided with the lack of AP-1 and NF- κ B activation (50). Whether tumor-specific T cells display impairment in transcriptional activity and if so, whether it represents a mechanism of generation of multiple defects of T-cell function, is not known.

2.2.3. ROLE OF APC-T-CELL INTERACTION

Several cytokine- and costimulatory molecule-dependent pathways are suspected to induce T-cell anergy in cancer. The interaction of the DC, the most potent APC, and T cells in induction and maintenance of the immune response is crucial for the generation

of functionally active CD4+ Th and CD8+ Tc cells. In murine tumors, a lack of CD40 ligation in DC-T-cell interaction may cause induction of anergy (51). Tolerance of tumor-specific CD4 T cells can be reversed with *in vivo* ligation of CD40 (52). Defective CD28 costimulatory pathway in tumor-bearing mice has also been shown to mediate the tumor-induced T-cell anergy (53). This suggests that defective APC-T-cell interaction-mediated signaling may be important for the induction of T-cell anergy in human cancers.

The role of defective interactions between APCs and T cells in cancer patients in the development of tumor-specific anergy has been demonstrated using acute myeloid leukemia-derived DCs. Precultured leukemic blast-derived CD1a+, CD83+ DCs induced significantly decreased proliferative responses of autologous T cells (54). This effect was attributed to tumor-derived DCs because mature nonleukemic DCs from the same patients were able to generate CTLs against autologous leukemia blasts. Whether membrane-associated or soluble factors are involved in this antigen-specific suppression of T-cell response mediated by tumor-derived DCs was not studied.

Animal studies show that such interactions can be mediated by regulatory cytokines. One of these cytokines, IL-10, is a suppressor cytokine of T-cell proliferative and Th1 (IL-2, IFN- γ) cytokine responses (55). IL-10 induces T-cell anergy by inhibiting the CD28 tyrosine phosphorylation, the initial step of the CD28-signaling pathway; this blocks binding of phosphatidylinositol 3-kinase p85 to CD28 costimulatory molecule (56). IL-10 blocks only T cells stimulated by low numbers of triggered T-cell receptors, a condition that is dependent on CD28 costimulation. T cells that receive a strong T-cell receptor signal and are, therefore, independent on CD28 cosignaling and resistant to the IL-10-mediated suppressive mechanism (55). However, tumor-associated antigens are weak antigens, and costimulation of T cells via cell surface-associated immunoregulatory molecules, such as B7 ligands of the CD28 receptor, seems to be obligatory for the generation of functionally competent T cells with antitumor activity. Whether the defective CD28-related mechanism is involved in T-cell anergy in cancer patients has not been elucidated.

IL-10-dependent mechanism of generation of anergic T cells may be different from the simple inhibition of cytokines that are necessary for the generation of potent antitumor CTL responses. Recently, IL-10-treated human DCs have been found to induce CD4+ and CD8+ T cells that suppressed antigen-specific proliferation of other T cells via cell-to-cell contacts (57). These cells did not induce an antigen-independent bystander inhibition and required activation by antigen-loaded DCs. These cells overexpressed CTLA-4 molecules, and blocking of the CTLA-4 pathway with antibodies significantly restored the proliferation of responder cells (57). These data demonstrate the “invasiveness” of antigen-specific negative signaling in anergy where inhibitory cytokine induces the ability of APCs to generate anergic cells that suppress other T cells. Whether the similar mechanism operates in cancer patients is not known.

A mechanism that involves another inhibitory pathway has been described in melanoma patients. Human melanoma-specific CTL and NK cells from normal donors (58) and melanoma patients (59–61) have been found to express CD94-NKG2A heterodimer, a receptor for MHC class I HLA-G and HLA-E. The engagement of CD94-NKG2A by tumor cell surface HLA-G (HLA-E) markedly inhibited both NK-cell and CTL activities whereas a blockade of CD94-NKG2A augmented the antitumor effects of NK cells and TCR- α/β + CD8+ CTLs (60,62). These cells are CD28-negative, strongly cytotoxic, and represent up to 10–80% of circulating CD8+ T cells in melanoma patients (60). A similar

mechanism involving negative signaling by CD94-NKG2A on CTLs has been described for the induction of susceptibility to a polyomavirus infection that induces tumors in mice (63). Whether the CD28– CTL phenotype and CD94-NKG2A inhibitory function operate in other cancer patients to induce T-cell anergy remains to be determined.

Continuous antigenic stimulation by tumor-associated antigens overexpressed in tumors may contribute to immune dysfunction in antigen-specific T cells in cancer patients, although this possibility should be examined in detail. Animal studies using transgenic mice demonstrated that the overexpression of a tolerizing antigen does not prevent the *in vitro* stimulation and recovery of T cells capable of mediating tumor destruction (64). Repetitive *in vitro* stimulation of memory T cells can recover anergic T cells and increase their antitumor activity (64). In breast cancer patients, bone marrow-localized memory T cells specific for tumor-associated antigenic epitopes can be stimulated using DCs and tumor lysates to generate significant antitumor CTL responses (65–67). This demonstrates the potential ability of anergic tumor-specific T cells to be stimulated by appropriate antigen-specific and nonspecific stimuli that should result in clinically relevant responses.

2.2.4. T-CELL DELETION BY APOPTOSIS IN CANCER

Most tumor-associated antigens are self-antigens that overexpressed in tumor cells. Mechanisms of the maintenance of immunological tolerance to self-antigens that hamper autoimmune reactions are also responsible for maintaining unresponsiveness to tumor cells. The existence of autoreactive T-cell clones, in both autoimmune diseases and cancer, however, demonstrates the abrogation of the control mechanisms that results in T-cell responses. In many cases, T cells ignore antigens if they are presented by nonspecialized APCs (68) in the absence of potent costimulation via CD28-B7 interaction (69). Recent studies provided evidence on the involvement of other membrane-associated costimulatory molecules in the generation of potent antitumor CTLs. One of these costimulatory molecules, 4-1BBL, interacts with its receptor, 4-1BB (CD137), which leads to the activation of CD4+ and CD8+ T cells via NF- κ B, JNK/SAPK, and p38 signaling pathways (70–72) and the induction of CTL responses against tumors (73). Costimulation by anti-CD137 antibodies converted a nontherapeutic CTL response elicited by the growing EL4E7 lymphoma in mice into a potent antitumor response (74). To eradicate the growth of another tumor cell line (*C3*) *in vivo*, additional peptide vaccination was needed (74). Mechanisms of immunopotentiation via CD137 may include, in addition to costimulation, the inhibition of T-cell apoptosis resulting in prolonged CTL responses (75,76). These data suggest that the absence or insufficiency of costimulation during interaction with tumor cells may result in a deletion of T cells whereas optimal costimulation *in situ* may overcome the induction of peripheral tolerance of T cells to epitopes from tumor-associated antigens.

T-cell apoptosis is a major mechanism for deletion of antigen-specific T cells following antigenic exposure (77), and it occurs frequently in cancer patients. Varying numbers of tumor-infiltrating T cells (5–20%) show DNA brakes as defined by an *in situ* TUNEL assay. Cell culture conditions dramatically increase apoptosis of isolated tumor-infiltrating lymphocytes (78,79). In the peripheral blood of patients with advanced melanoma and gastric cancer, the spontaneous apoptosis of a small subpopulation of T cells has been observed (40,80,81). Activation of peripheral T cells isolated from cancer patients significantly increased the proportion of apoptotic cells.

One of the mechanisms of T-cell deletion involves the interaction of CD95 (Apo-1/Fas) on T cells and its ligand (CD95L) that is expressed in tumor cells. Jurkat T cells that express CD95 receptor underwent a CD95L-specific apoptosis when interacted with CD95L-positive breast cancer cells (82). Several breast cancer cell lines were found to upregulate the expression of CD95L by IFN- γ and induced an increased apoptosis of interacting T cells in the presence of this cytokine (82). Authors have suggested that this mechanism might favor the immune escape of breast cancer cells in the presence of IFN- γ -producing tumor-infiltrating lymphocytes. Further, CD95L easily shed from the tumor surface to the supernatant, which acquires the capability to induce apoptosis of CD95-positive cells (82). Depletion of CD4+ and CD8+ T cells in peripheral blood in breast cancer patients positively correlated with the CD95L expression in the tumors, providing evidence for possible role of CD95L in systemic immune suppression that represents another mechanism of the tumor escape. Although the CD95L/CD95 pathway appears to play a role in the deletion of activated tumor-reactive T cells in different cancers, some specific tumors do not express CD95L. One example is melanomas that do not express CD95L while melanoma-specific T cells are induced to undergo apoptosis following MHC class I-restricted recognition of the tumor antigen in a CD95-dependent fashion (83). Peripheral blood of some cancer patients contains T cells expressing the CD95 receptor (80), and spontaneous apoptosis of T cells may also be mediated via the CD95/CD95L pathway (80,81). Other tumor-derived products such as gangliosides can increase the sensitivity of T cells to activation-induced cell death in the tumor microenvironment (79). It is unclear, however, whether the deletion of T cells by activation-induced cell death in blood or the tumor site represents a mechanism of maintenance and/or the induction of tumor antigen-specific immunological nonresponsiveness.

2.3. Th1- vs Th2-Type Response in Cancer

In animal studies, the production of Th1 cytokines IFN- γ and IL-2 by CD4+ T cells is linked to the generation of therapeutically efficient immune responses that reject tumors (84). IL-2 is required for T-cell proliferation and the acquisition of cytotoxic effector function. On the other hand, type 2 cytokines, IL-4 and IL-10, promote Th2 responses that are necessary for the induction of a humoral immune response.

In animal models of cancer and cancer patients, the tumor microenvironment can swing the Th1/Th2 balance to a predominantly Th2 response. Such predominance has been demonstrated in the peripheral blood of patients with advanced-stage cancer (85,86). The measurement of intracellular levels of cytokines in renal cell carcinoma patients by flow cytometry demonstrated a significant shift from a Th1 to a Th2 response with an increasing stage of the disease (87). A Th2 bias and barely or undetectable IFN- γ expression by CD4+ T-cell population in cancer might create an environment that would not support the development of tumor antigen-specific CD4+ T cells.

Although factors causing a shift to Th2 responses in cancer patients are not well defined, animal studies suggest that both TGF- β and IL-10 are involved in this process. TGF- β may skew T cell responses to Th2 via IL-10 as an intermediate and/or inhibit Th1-type responses directly in certain animal models (88,89). IL-10 downregulates the expression of Th1 cytokines and amplifies the immunosuppressive activity of TGF- β by the upregulation of TGF- β type II receptors (84,90). The contribution of IL-10 to immunosuppression in animal models of cancer is complicated by observations indicating that

IL-10 may promote antitumor immunity by decreasing tumor burden through the inhibition of tumor neovascularization.

Other data also seem to contradict the concept that only IFN- γ -producing T cells may have a therapeutic impact in cancer. Both type 1 (Tc1) and type 2 (Tc2) CD8+ isolated effector T cells adoptively transferred in mice bearing an established pulmonary tumor were shown to promote long-term tumor immunity and protection against syngeneic B16 tumor (91). The possible role of excessive production of IL-10 by Tc2 cells that might result in inhibition of neovascularization was not determined in this study. Another study utilized isolated tumor-specific CD8+ T cells activated in vitro to generate CTLs secreting either IFN- γ and no IL-4 and IL-5, either both IL-4 and IL-5 and some IFN- γ . Both cell types killed target cells in vitro (92). Following transfer, only type 1 (Tc1) CD8+ T cells significantly protected against tumor challenge, whereas type 2 T cells (Tc2) had no effect. Although slightly contradictory, most available data in mice confirm the predominant role of type 1 T-cell responses in tumor protection and emphasize that both Th1 and Tc1 cells secreting IFN- γ might have an impact on cancer treatment and/or protection. Interestingly, favorably increased Th1/Th2 and Tc1/Tc2 ratios were induced shortly after debulking surgery in patients with lung carcinoma as determined in tumor-infiltrating lymphocytes by intracellular IFN- γ and IL-4 production with three-color flow cytometry (93). In contrast, tumor recurrences reversed these ratios in peripheral blood of cancer patients confirming that cancer progression is associated with the shift to type 2 T-cell responses in both CD4+ and CD8+ T-cell populations (93).

In cancer patients, there are correlative data linking increased expression of type 2 cytokine IL-10 and TGF- β and preferential type 2 T-cell phenotype. In pancreatic carcinoma patients, coexpression of TGF- β and IL-10 in tumor tissue was associated both with elevated levels of cytokines in the sera and with the predominance of a type 2 T-cell cytokine pattern in response to anti-CD3 antibody stimulation (86).

Animal tumor model studies and clinical studies revealed the value of determination of type 1/type 2 T-cell cytokines in blood, lymph nodes, and intratumorally and emphasized the IFN- γ expression as the most valuable marker of antitumor responses. Recent and future advances in RNA and protein array technology should help to define the most informative patterns of multiple cytokine gene expression in tumors. This might be particularly useful for the monitoring and prediction of outcome following immunotherapeutic approaches such as vaccination against cancer.

3. DEFECTIVE DENDRITIC CELL DIFFERENTIATION AND FUNCTION IN CANCER

3.1. *Abnormal Dendritic Cell Differentiation and Accumulation of Immature Myeloid Cells in Cancer*

The induction of an effective antitumor immune response requires antigen presentation by host APCs (94). DCs are the most potent APCs. DCs, macrophages, and granulocytes are differentiated from common myeloid progenitors. Only mature DCs can efficiently prime CD8+ CTL precursors (95). Impaired differentiation of the myeloid lineage may result in the accumulation of cells able to inhibit CD8+ T cells (96). Tumors severely affect differentiation of myeloid cells, which results in the decreased production of mature DCs resulting in low DC numbers in peripheral blood and lymph nodes of cancer patients as well as tumor-bearing mice. A direct correlation between DC infiltration

tion of tumor and outcome of the disease has been established. A decreased DC production in cancer is associated with the accumulation of immature myeloid cells. These cells express class I MHC molecules and inhibit antigen-specific responses of CD8+ T cells but not CD4+ T cells (97) via direct cell-to-cell contact (98). In mice, these immature cells have a phenotype of CD11b+GR-1+ cells. Murine carcinomas such as Lewis lung carcinoma (99), CT-26 (100), MCA-26 (101), Erlich carcinoma (102), mammary adenocarcinoma D1-DMBA-3 (103), MethA, C3 (97), and CMS5 (13) sarcomas are all associated with splenomegaly, extramedullary hematopoiesis, and the significant accumulation of immature myeloid cell in the spleen and at some degree in lymph nodes.

Similar findings have been reported in cancer patients. An accumulation of immature myeloid cells lacking markers for mature cells of myeloid and lymphoid lineages was associated with the decreased numbers of DCs in the peripheral blood of cancer patients (104). Advanced-stage cancer promoted the accumulation of these cells in blood whereas surgical resection of the tumor decreased the presence of immature cells. This was consistent with the data reported for the mouse tumor model (13).

Functional activity of immature myeloid cells involves the inhibition of interferon- γ production by CD8+ T cells in response to a specific peptide presented by MHC class I in vitro and in vivo (97). This effect was not mediated by soluble factors, and a blockade of MHC class I molecules on the surface of Gr-1+ cells completely abrogated the inhibition. To investigate whether immature myeloid cells obtained from cancer patients affect MHC class I-restricted responses, an HLA-A2+ CTL line specific for an influenza virus-derived peptide was generated (104). Incubation of these cells with Lin-HLA-DR-immature myeloid cells isolated from the peripheral blood of HLA-A2-positive cancer patients inhibited the production of IFN- γ by CD8+ T cells stimulated by peptide-pulsed DCs. A similar mechanism of immunosuppression was described in mice (96,105). Antigen-specific restimulation in vitro of CD8+ T cells obtained from mice immunized with a tumor antigen resulted in apoptosis of CD8+ T cells and the abrogation of CTL activity whereas the depletion of Gr-1+ cells prevented the apoptosis and restored the CTL activity. The induction of apoptosis of CD8+ T cells was mediated by spontaneously generated adherent Gr-1+ F4/80+ cells (96).

Although the phenotype of tumor-induced human suppressor cells of myeloid origin was determined as Lin- HLA-DR-, other hematopoietic cells may also be responsible for immunosuppression in cancer patients. Profound defects in T-cell function in patients with head and neck squamous cell carcinoma were found to be associated with the increased levels of CD34+ cells (marker of late hematopoietic cell progenitors) (106,107). The depletion of these cells restored the proliferative responses of T cells to IL-2. Whether the heterogeneous suppressor cells utilize the same or different molecular mechanisms of immunosuppression, remains to be elucidated.

3.2. Tumor-Derived Factors Involved in Defective DC Differentiation

Several cytokines produced by tumors have been implicated in the defective DC maturation and the impairment of myelopoiesis. One of these factors is vascular endothelial growth factor (VEGF), which is produced by most tumors, and its production is a marker of poor prognosis. VEGF stimulates the formation of tumor neovasculature (108). Anti-VEGF neutralizing antibodies block the negative effects of tumor cell supernatants on DC maturation in vitro (109). The expression of VEGF negatively correlated with the DC numbers in tumor (110) and peripheral blood (111,112) of cancer patients.

The administration of recombinant VEGF to naïve mice resulted in an inhibition of DC development and was associated with an increase in production of immature Gr-1+ myeloid cells (113).

Another factor, which is involved in the accumulation of immature myeloid cells in cancer, is granulocyte macrophage colony-stimulating factor (GM-CSF). About 30% of 75 tested human tumor cell lines spontaneously secrete this cytokine (114). The administration of anti-GM-CSF and anti-IL-3 antibodies *in vivo* abrogated the accumulation of immature cells in mice bearing Lewis lung carcinoma. Multiple injections of GM-CSF in mice resulted in the generation of a cell population that expressed the granulocyte-monocyte markers Mac-1 (CD11b) and Gr-1 (114). These Gr-1/Mac-1 double-positive cells can be differentiated *in vitro* into mature and fully functional APCs in the presence of IL-4 and GM-CSF. It is likely that both GM-CSF and VEGF may significantly contribute to tumor escape via the induction of suppressor cells.

Other tumor-derived factors such as M-CSF, IL-6, IL-10, and gangliosides also contribute to defective DC differentiation *in vitro* (115–119). IL-10 prevented the differentiation of monocytes to DCs but promotes their maturation to macrophages (116). DCs derived from transgenic mice overexpressing IL-10 significantly suppressed MHC alloreactivity, CTL responses, and IL-12 production (120).

3.3. Molecular Mechanisms of DC Dysfunction in Cancer

Cell differentiation is a complex process, which involves multiple genes controlled by multiple transcription factors. One of the transcription factors, NF-κB, is especially important for DC biology. NF-κB regulates the transcription of many genes involved in immune responses (reviewed in refs. 121 and 122). NF-κB is composed of 50- and 65-kDa subunits (p50, p52, p65 [RelA], cRel, and RelB), which are bound to a 10-bp motif in the promoter of responsive genes. These subunits form both homo- and heterodimeric complexes and differentially regulate gene expression (123). NF-κB is present as an inactive complex in the cytoplasm of many cells bound to members of the IκB family of inhibitory proteins. Activation of NF-κB involves serine phosphorylation, dissociation, and degradation of IκB followed by release and nuclear translocation of NF-κB. Several studies have demonstrated that RelB, a component of NF-κB, is required for the development of DCs (124–126). Block of NF-κB with dominant inhibitor IκBα prevented DC differentiation from progenitor cells *in vitro* (127). More recently Wu et al. have confirmed that RelB is essential for the development of myeloid DCs (128).

Tumor cell-conditioned media and VEGF inhibited the activation of NF-κB in hematopoietic progenitor cells *in vitro* (127). This inhibition was seen as early as 2 h after the treatment with tumor cell supernatants or VEGF. Similar results were obtained *in vivo*. NF-κB activation was blocked in bone marrow cells as early as 7 d after the start of VEGF infusion. This well preceded any morphological changes observed in bone marrow of these mice (113). The same inhibitory effect was detected in tumor-bearing mice (113). These data indicate that inhibition of NF-κB could be a mechanism responsible for a defective DC differentiation in cancer.

The nature of the genes responsible for these effects remains unknown. Recently, using VEGF as a natural inhibitor of DC development, we sought to identify genes essential for differentiation of these cells. Using a differential display assay (DDA) we have found that H1^o histone is one of the genes specifically inhibited by VEGF in hematopoietic progenitor cells (HPCs). The H1^o histone is a lysine-rich member of the H1 family

of linker histones. These proteins bind to the linker DNA between nucleosome cores and facilitate the formation of higher order chromatin structures (129). Considerable evidence connects histone H1 to gene regulation (130–132). We have found a close association between expression of this histone and DC differentiation *in vitro*. DC production in H1^o-deficient (*h1^{o/-}*) mice was significantly decreased (133). Tumor-derived factors considerably reduced *h1^o* expression in HPCs. We have demonstrated that transcription factor NF-κB is actively involved in regulation of *h1^o* (133). Generation and function of macrophages, granulocytes, and lymphocytes appears to be normal in H1^o-deficient mice. However, these mice had significantly lower response to vaccination with specific peptide or ovalbumin than control mice (133). These data indicated that H1^o histone might be an important factor for normal DC differentiation. Tumor-derived factors may inhibit DC differentiation by affecting H1^o expression.

Currently, autologous DCs represent an optimal vehicle for the delivery of tumor vaccines to the immune system of the cancer patient. The potential therapeutic benefit of this intervention, however, may be limited because of the significant proportion of immunosuppressive immature myeloid cells present in the tumor-bearing host. One approach to correct this problem might include the differentiation of immature myeloid cells using growth factors and compounds that promote the differentiation of immature cells. We suggest that all-trans retinoic acid (ATRA) can be used both *in vitro* and as an injection to delete immunosuppressive DCs. In cancer patients, ATRA has been shown to significantly abrogate immature myeloid cell numbers and promote human DC differentiation *in vitro* (104). Similar effects were observed in tumor-bearing mice (97). Another approach includes the prevention of the accumulation of immature DCs. Our preliminary data (D. Gabrilovich) demonstrate the role of reactive oxygen species in the generation of immature DCs with the immunosuppressive ability. A blockade of some of these species with antioxidants may result in a decreased generation of immunosuppressive immature myeloid cells and DCs and ultimately improve the therapeutic effect of cancer vaccines.

4. NATURAL KILLER CELL DEFECTS IN CANCER

NK cells are cytotoxic effector cells that are involved in the lysis of a tumor (134). These cells produce IFN-γ and TNF-α, which further stimulate both T cells and NK cells. Three main mechanisms are responsible for the cytotoxic function of NK cells against tumor targets. These mechanisms include the CD95L-CD95 (Fas ligand-Fas) pathway (135,136), TNF-related apoptosis (137,138), and granule exocytosis. The latter involves the release of a pore-forming protein, perforin (139), and apoptotic proteases, granzymes (140), which are released from the cytotoxic granules of NK cells. Perforin targets cell pores and allows entry of NK-cell-derived granzymes into the cytoplasm of the tumor target cell that results in a cytotoxic effect (141). One of the main cytotoxic molecules that induces cytotoxic effect against different tumor lines is granzysin (142,143).

Tumor progression is associated with the impairment in cell number or function of NK cells (144,145). Metastases in head and neck cancer patients were also found to be dependent on NK-cell activity (146). The NK-cell defects related to cancer involve a decrease of both activity and numbers of CD56+ and CD56^{dim} NK cells (147,148), impaired perforin-dependent mechanism of cytotoxicity (metastatic melanomas) (149), and immunosuppression associated with impaired granzysin expression (150). Patients

with advanced lung, breast, gastrointestinal, hepatobiliary, pancreatic, urinary tract, uterine, and ovarian cancers had significantly fewer granulysin-positive NK cells than healthy individuals. The expression of perforin determined in this study was not significantly impaired (150). In some cases, the defective function of NK cells was associated with low level of spontaneous IFN- γ secretion (151). The mechanisms of NK-cell defects in cancer patients can also be linked to abnormally high levels of immunosuppressive molecules secreted by tumor or monocytes/macrophages (TGF- β , IL-10, IL-6, and others) or be mediated by the generation of reactive forms of oxygen, particularly hydrogen peroxide, overproduced in monocytes and macrophages in cancer. Phorbol diester tumor promoter, TPA, induced reactive forms of oxygen in NK cells and suppressed their function, whereas catalase, the hydrogen peroxide scavenger, reversed NK-cell suppression (152). Whether this mechanism is operated in cancer patients remains to be elucidated. The direct inhibition of NK cells by tumor cells was demonstrated in experiments that used renal cell carcinoma cell lines sensitive to CD56+ NK cells. These tumor cells inhibit NK cells via mechanism dependent on the CD94 receptor (153).

Another source of low function of NK cells may derive from insufficient costimulation by DCs. DCs are able to augment NK-cell function (154). Human DCs enhance proliferation, effector function, and survival of NK cell lines (155) or NK cells from patients with glioblastomas (156) via cell-to-cell contact, even in the absence of exogenous cytokines. This suggests that the improvement of DC function in a cancer patient may also result in an improvement of NK-cell activity. This issue need to be confirmed both in experimental and clinical settings.

5. CONCLUSIONS

Tumors developed a number of mechanisms to avoid recognition and elimination by the host immune system. Probably, the first one is immunological ignorance. It is taking place when the number of tumor cells and the amount of tumor-associated antigens available are too little to be recognized by the host immune system. When the tumor grows bigger it employs several new mechanisms. Lack of inflammation at that stage does not support the immune system in the identification of tumor-associated antigens as “foreign,” which probably results in a continuous state of tumor-specific tolerance. It also helps that most of those tumor-associated antigens are self-proteins. In addition, the tumor itself produces cytokines and growth factors able to suppress differentiation and activation of DCs and T cells, and stimulates the generation of immature myeloid cells. All together this promotes the state of antigen-specific nonresponsiveness (tolerance). In the later stage of tumor growth, an increased production of tumor-derived factors results in more or less prominent systemic immunosuppression, which manifests in a T-cell inability to produce cytokines or respond to antigenic or mitogenic stimuli, in accumulation of immature myeloid cells, and depletion of professional APCs (DCs). In addition, tumor cells may directly eliminate T cells via CD95/CD95L (Fas/FasL) or soluble factors-mediated apoptosis. All of this makes an adequate immune response to tumor impossible. It also undermines the effects of cancer vaccines. The success of cancer vaccines depends on the adequate function of the host immune system. The removal of bulky disease by surgery, chemo-, or radiation therapy prior to vaccination appears to be necessary for the success of vaccination. In addition, the elimination of immunosuppressive myeloid cells by their differentiation as well as support of T cells through the delivery of

exogenous cytokines may dramatically improve the ability of immune system to respond to vaccination. Of course, adequate function of the immune system does not guarantee the success of the immunotherapy of cancer. Even in the presence of a strong immune response, a tumor may avoid elimination by downregulating MHC class I or tumor-associated antigens, which would argue in favor of multiepitope vaccines.

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7

Escape of Tumors From the Immune System *Role of the Transforming Growth Factor- β -Signaling Pathway*

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1. INTRODUCTION

It is widely accepted that tumors may evade the immune response by a number of different mechanisms described in Chapter 6 including: antigenic variation, downregulation of tumor-specific antigens, downregulation of major histocompatibility complex (MHC) molecules, and lack of sufficient costimulation to T cells by tumors that are considered as “self.” Mechanisms by which tumors may suppress the immune system include: interference with antigen processing and/or presentation, induction of apoptosis in lymphocytes by Fas ligand (FasL) or other “death” receptors, interference with the ability of leukocytes to migrate into the tumor, and active tumor-mediated immunosuppression. Although support for all of these mechanisms exists in experimental models and/or specific tumor types, there is mounting evidence that active tumor-mediated immunosuppression may be the most general and potent way for human tumors to escape the immune system. In addition, current evidence supports a model by which this active

tumor-mediated immunosuppression is largely the result of secretion of the potent immunosuppressive cytokine, transforming growth factor- β (TGF- β). In this review, the evidence supporting such a role for TGF- β and the mechanisms by which TGF- β may be acting to suppress the immune system are discussed. Finally, strategies to target the TGF- β -signaling pathway to ultimately improve the success of immunotherapy for human cancers are explored.

2. OVERVIEW OF THE TGF- β -SIGNALING PATHWAY

Although the TGF- β -signaling pathway has been the subject of many excellent recent reviews (1–3), to facilitate discussion of the manner in which this pathway could be manipulated in a clinically relevant fashion, the current state of knowledge of the TGF- β -signaling pathway is outlined.

TGF- β is a member of a superfamily of dimeric polypeptide growth factors that includes bone morphogenetic proteins, activins, and inhibins. All of these growth factors share a cluster of conserved cysteine residues that participate in intramolecular disulfide bonds and form a common cysteine knot structure (1). Virtually every cell in the body produces at least one isoform of TGF- β , has receptors for TGF- β on their cell surface, and responds to TGF- β stimulation in some fashion. In addition to its role in regulating the immune system as detailed below, TGF- β has many additional physiological roles including regulating cellular proliferation, differentiation, and apoptosis in a diverse array of cell types, as well as regulating complex processes such as development, wound healing, and angiogenesis. The vital role of the TGF- β -signaling pathway in these cellular processes has been demonstrated by targeted deletion of the genes encoding members of the pathway in mice as has been reviewed elsewhere (4).

Particularly relevant to the present discussion is the biphasic effect of TGF- β on tumorigenesis. The TGF- β -signaling pathway is involved in tumorigenesis both as tumor suppressors and as tumor promoters. After tumor cells themselves become resistant to the tumor suppressor activities of TGF- β (through regulation of cellular proliferation, differentiation, and apoptosis), either by mutation in or loss of expression of TGF- β -signaling molecules, both stromal cells and tumor cells increase their production of TGF- β ligands. In response to increased production of TGF- β , the tumor cells (which can no longer be growth inhibited) become more invasive and metastasize to distant organs as a result of the effects of TGF- β as a tumor promoter (through regulation of cellular invasion, metastasis, angiogenesis, and the immune system). Thus, resistance to TGF- β leads both to conversion to malignancy (TGF- β as a tumor suppressor) and to a more malignant phenotype (TGF- β as a tumor promoter). This dichotomous role of TGF- β in tumorigenesis remains a fundamental problem, in terms both of our understanding of the TGF- β -signaling pathway, and the ability to target this pathway for the chemoprevention or treatment of human cancers.

TGF- β exists as three isoforms in mammals, TGF- β 1, TGF- β 2, and TGF- β 3, each of which is encoded by a unique gene and expressed in a tissue-specific and developmentally regulated fashion. In addition, all three isoforms are highly conserved, suggesting a critical biological function for each isoform. Indeed, these TGF- β isoforms differ in their binding affinity for TGF- β receptors (e.g., TGF- β 2 binds the type III TGF- β receptor, but not the type II TGF- β receptor), and when deleted in mice result in unique phenotypes.

All TGF- β isoforms are synthesized as part of a large precursor containing a propeptide region and a region encoding mature TGF- β . TGF- β is processed from this precursor by cleavage of the propeptide from mature TGF- β prior to secretion from the cell. However, mature TGF- β remains noncovalently attached to the propeptide. Upon secretion, most TGF- β is stored in the extracellular matrix as a latent complex (containing TGF- β , the noncovalently bound propeptide, and disulfide bond-bound latent TGF- β -binding proteins), which prevents TGF- β from binding to its receptors. The mechanism by which mature, biologically active TGF- β is released from these latent complexes *in vivo* remains poorly understood, but may involve the action of thrombospondin-1, which acts by changing the conformation of the latent TGF- β -binding protein (5), or plasmin-mediated proteolytic cleavage of the complex. As most cells secrete TGF- β and express its receptors, activation of TGF- β represents a critical regulatory step in TGF- β action, one that could potentially be targeted in a clinically relevant manner.

TGF- β regulates cellular processes by binding to three high-affinity cell-surface receptors, the type I, type II, and type III TGF- β receptors (Fig. 1). The type III receptor (T β RIII) is ubiquitously expressed and is the most abundant TGF- β receptor, with approximately 100,000–300,000 copies/cell. T β RIII classically functions by binding the TGF- β ligand and then transferring it to the signaling receptors, the type I TGF- β receptor (T β RI) and type II TGF- β receptor (T β RII). Although this “presentation” role may be one function for T β RIII, particularly relevant to TGF- β 2, which cannot bind T β RII independently, recent studies by our group and others have suggested a larger role for this receptor. Specifically, T β RIII has been shown to have an essential nonredundant role in TGF- β signaling, mediating the effects of TGF- β (TGF- β 1 or TGF- β 2) on mesenchymal transformation in chick embryonic heart development (6). In addition, the loss of functional T β RIII expression on intestinal goblet cells is sufficient to mediate resistance to TGF- β (7). Our laboratory has determined that although T β RIII lacking its cytoplasmic domain can bind TGF- β , associate with T β RII, and present TGF- β to T β RII, its ability to mediate TGF- β 2 signaling in a biological assay is impaired (8). Our laboratory has further defined specific functions for the cytoplasmic domain of T β RIII, namely phosphorylation by T β RII, associating specifically with the cytoplasmic domain of autophosphorylated T β RII, dissociating T β RIII from the signaling complex between T β RII and T β RI, and binding the PDZ domain-containing protein, GIPC (8). GIPC expression enhances T β RIII expression at the protein level and this enhanced expression is sufficient to enhance TGF- β signaling (TGF- β 1 and TGF- β 2) (9). More recently, T β RIII has been demonstrated to have a role in inhibiting TGF- β signaling in LLC-PK1 porcine epithelial cells (10). This role is not dependent on the cytoplasmic domain of T β RIII, but instead depends upon post-translational modification of the extracellular domain (10). In addition, T β RIII has the potential to be proteolytically cleaved in the extracellular domain immediately adjacent to the transmembrane domain, producing a soluble extracellular domain of T β RIII (sT β RIII). sT β RIII antagonizes TGF- β signaling, at least in part, by sequestering TGF- β ligand. Thus, the effects of T β RIII on TGF- β signaling may be positive or negative depending on the level of expression, the extent of proteolytic processing, and the cellular context of the receptor. Taken together, these studies are defining essential and complex roles for T β RIII in regulating TGF- β signaling in both a positive and negative manner, and suggest that regulating T β RIII expression and/or processing represents an attractive method for regulating TGF- β signaling.

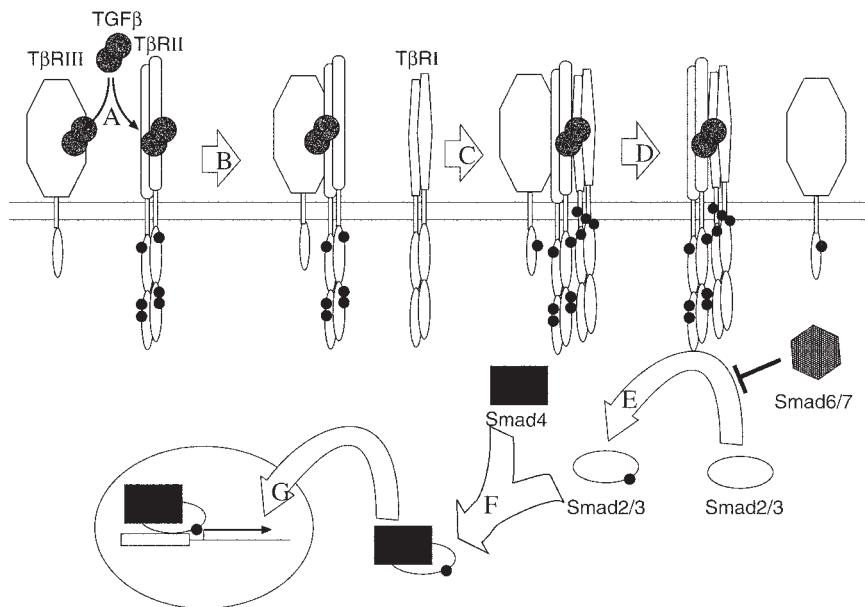


Fig. 1. Mechanism of TGF- β mediated signal transduction. TGF- β ligand binds to T β RIII (**A**) and T β RII preferentially presents TGF- β to the autophosphorylated type II receptor (T β RII) via a specific interaction of the cytoplasmic domain of T β RIII with the cytoplasmic domain of autophosphorylated T β RII (**B**). TGF- β may also bind T β RII directly (**A**). The complex of TGF- β , T β RIII, and T β RII recruits and binds T β RI (**C**). T β RII transphosphorylates and activates T β RI, and transphosphorylates the cytoplasmic domain of T β RIII releasing T β RIII from the active signaling complex of T β RI and T β RII (**D**). The active signaling complex containing activated T β RI then phosphorylates Smad2 or Smad3 (**E**). Phosphorylated Smad 2 or 3 binds to Smad 4 (**F**), the common Smad, and the resulting complex translocates from the cytoplasm into the nucleus (**G**). In the nucleus, the Smad complex interacts in a cell-specific manner with various other transcription factors to regulate either positively or negatively the transcription of TGF- β -responsive genes and mediate TGF- β effects at the cellular level. Inhibitory Smads 6 and 7 lack the region normally phosphorylated by T β RI and thus act by interfering with phosphorylation of Smad 2 or 3 by T β RI. Phosphorylation sites/events are signified by the black circles.

T β RI and T β RII initiate intracellular signaling by phosphorylating several transcription factors, the Smads (derived from the Sma [for small] and MAD [for mothers against de^capentaplegic] gene homologs in *Caenorhabditis elegans* and *Drosophila melanogaster*) via the serine/threonine protein kinases located in their intracellular domains. The TGF- β superfamily signals through ten Smad proteins, with Smads 2 and 3 being downstream of T β RI. Smad 4 is a common partner for all of the receptor-activated Smads, whereas Smads 6 and 7 are inhibitory Smads that block the phosphorylation of Smad 2 or 3, thus inhibiting TGF- β signaling. TGF- β has also been demonstrated to act through the mitogen-activated and stress-activated protein kinase pathways, although the precise mechanism and the physiological relevance of this signaling has not been established (11).

Taken together these studies have established a framework for TGF- β signaling as outlined in Fig. 1 (12,13). Signaling is initiated by TGF- β , which binds either to T β RIII,

which then presents TGF- β to T β RII (essential for TGF- β 2), or to T β RII directly. Once activated by TGF- β , T β RII recruits, binds, and transphosphorylates T β RI, thereby stimulating their protein kinase activity. T β RII may also phosphorylate T β RIII, releasing it from the complex of T β RII and T β RI, allowing downstream signaling to occur (8). The activated T β RI then phosphorylates Smad 2 or 3, which forms a complex with Smad 4, and the resulting complex translocates into the nucleus where it interacts in a cell-specific manner with various transcription factors to regulate the transcription of many TGF- β -responsive genes.

3. ACTIVE TUMOR-MEDIATED IMMUNOSUPPRESSION: MECHANISMS OF TGF- β ACTION

As discussed above, secretion of TGF- β by tumors and the surrounding stroma cells is perhaps the most widely used method by which tumors actively suppress the immune system. The immunosuppressive effects of TGF- β have been demonstrated in vitro and in vivo, with exogenous TGF- β 1 producing immunosuppression in animal models (14). How does TGF- β mediate its immunosuppressive effects? Although TGF- β has diverse effects on virtually every cell type in the immune system, for the purposes of this review, the focus will be on its immunosuppressive effects on cells involved in tumor immunology, i.e., T cells and antigen-presenting cells (APCs). The effects of TGF- β on other aspects of the immune system have been recently reviewed (15).

TGF- β was first found to have a role in T-cell biology through its production by T cells and its role in inhibiting the interleukin-2 (IL-2) dependent proliferation of T cells (Fig. 2; 16). Independent of this antiproliferative effect, TGF- β also inhibits the differentiation of T cells, and in general, prevents naïve T cells from acquiring effector functions (to become cytotoxic T lymphocytes [CTLs] or T-helper cells under the appropriate circumstances) (17). TGF- β may mediate some of its immunosuppressive effects on T cells through secretion by and cell surface expression on CD4+CD25+ regulatory T cells (18). These in vitro effects have been validated in murine models. For example, TGF- β 1-deficient mice develop a severe autoimmune phenotype leading to death by 3 wk, resulting in part from overactive T cells (19). In addition, T-cell-specific abrogation of TGF- β signaling in mice (by expression of either the inhibitory Smad, Smad7 (20) or dominant-negative T β RII (21,22)) results in spontaneous T-cell activation, with the mice developing an autoimmune disease of the lung and colon. These results demonstrate that TGF- β signaling is required for T-cell homeostasis in vivo.

TGF- β also has potent effects on professional APCs. TGF- β is produced by macrophages and has been demonstrated to inhibit their activation, particularly tissue macrophages (Fig. 2; 23). TGF- β also appears to be required for differentiation of dendritic cells from precursors, primarily by protecting their viability (24). Though TGF- β may be responsible for differentiation of dendritic cells from CD34+ precursors, it is also responsible for preventing the maturation of dendritic cells (25). In terms of the effects of TGF- β on dendritic cells in vivo, TGF- β 1-deficient mice are again instructive. These mice have a complete absence of Langerhans cells in the epidermis, although their bone marrow contain functional precursors that can form Langerhans cells in other hosts, suggesting that TGF- β is required for normal Langerhans-cell development and/or migration to the epidermis (26).

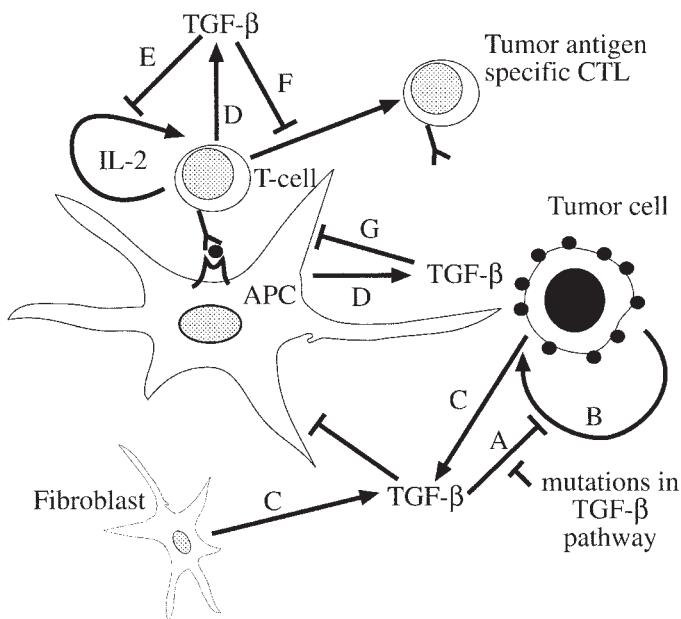


Fig. 2. Role of TGF- β in tumorigenesis and cancer-mediated immunosuppression. In normal cells, TGF- β acts as a tumor suppressor to inhibit cellular proliferation (**A**). During tumorigenesis, various components of the TGF- β -signaling pathway are mutated, making the tumor cells resistant to the effects of TGF- β . These TGF- β -resistant tumor cells, which proliferate in an unregulated manner (**B**), as well as the surrounding stromal cells (fibroblasts), then increase their production of TGF- β (**C**). In addition, immune cells infiltrating the tumor (T cells and APCs) also produce TGF- β (**D**). This TGF- β then acts to inhibit the proliferation of the tumor-infiltrating T cells (**E**) as well as their conversion to CTLs (**F**). In addition, TGF- β inhibits the maturation and activation of APCs (**G**). Together, these effects of TGF- β result in potent immunosuppression, particularly in the tumor milieu, partially mediating the tumor-promoting effects of TGF- β .

In addition to its direct effects on T cells and APCs, TGF- β may also mediate effects on T cells and APCs via stimulation of other immunosuppressive agents, including IL-6 or IL-10.

4. EXPERIMENTAL EVIDENCE SUPPORTING A ROLE OF TGF- β IN ACTIVE TUMOR-MEDIATED IMMUNOSUPPRESSION

The evidence presented above supporting TGF- β as a major mechanism for tumor-mediated immunosuppression is primarily circumstantial. If TGF- β is the major mechanism for tumor-mediated immunosuppression, one would predict that blocking the TGF- β -signaling pathway would result in more effective tumor vaccines, and conversely, that increased TGF- β signaling would allow tumors to escape the immune system. These predictions have been experimentally verified.

In terms of increasing TGF- β signaling, expression of TGF- β 1 in the highly immunogenic CH-3 cell line (to increase the level of TGF- β 1 in vivo) allows this cell line to escape immune surveillance and form tumors in mice, whereas the non-TGF- β 1-expressing cell line cannot (27). Importantly, there is no downregulation of MHC class I molecules or tumor-specific antigens in this model. In addition, overexpression of TGF- β 1 (by either

transfection with a TGF- β 1 expression vector or administration of TGF- β 1 protein intra-peritoneally) in the MCF-7 breast cancer cell line allowed tumor formation in an estrogen-independent fashion (28).

Strategies to block the TGF- β -signaling pathway include decreasing expression of TGF- β ligand with antisense to TGF- β , decreasing TGF- β ligand binding to responsive cells with antibodies to TGF- β ligand or expressing the soluble extracellular domain of T β RII, or expressing a mutant form of the T β RII that lacks the entire kinase domain and most of the juxtamembrane region, T β RII Δ cyto, which acts to block TGF- β signaling in a dominant-negative fashion (29). Initial studies performed using antibodies to TGF- β ligand were able to enhance tumor-specific immune responses and reduce tumorigenicity, but not alter the ability of the immune system to eradicate the tumor (30,31). However, neutralizing antibodies to TGF- β (prevents immunosuppression) combined with IL-2 therapy (to stimulate the immune system) was able to decrease the number of metastases in a murine B16 melanoma model (32). Subsequent studies, performed by inserting an antisense construct to TGF- β ligand in resected tumors and then vaccinating with those cells, successfully increased the ability of these vaccines to eradicate tumors (33,34). In addition, overexpression of soluble T β RII in the mouse thymoma cell line EL4, suppressed the tumorigenicity of this tumor *in vivo*, along with increasing tumor-specific CTL activity, indicating that the suppressed tumorigenicity was mediated by preventing TGF- β -mediated immunosuppression (35). Importantly, blockade of the TGF- β -signaling pathway specifically in CD4+ and CD8+ T cells through expression of T β RII Δ cyto in these cells, has recently been shown to increase the ability of these T cells to produce specific antitumor (thymoma and melanoma) cytotoxic responses and to eradicate these tumors *in vivo* (36).

Finally, studies examining the effects immunosuppression of multiple myeloma-derived TGF- β suggested that specific populations of T cells may be resistant to the effects of TGF- β . Indeed, treatment with IL-15 was able to abrogate tumor-derived TGF- β -mediated immunosuppression *in vitro* (37).

5. FUTURE STRATEGIES TO CIRCUMVENT ACTIVE TUMOR-MEDIATED IMMUNOSUPPRESSION VIA TGF- β

Although the above results are intriguing, and suggest that manipulating the TGF- β -signaling pathway represents an attractive means of enhancing current vaccine strategies, each of the current approaches has potential limitations. In general, these approaches are generic, acting to globally block TGF- β signaling, which increases the potential for deleterious effects, particularly when utilized in cancer patients. As discussed above, TGF- β has a biphasic effect on tumorigenesis. Thus, if TGF- β action is globally suppressed, one could potentially promote the formation of other tumors or increase the tumorigenicity of tumors that are not already TGF- β -resistant. For example, blockade of the TGF- β -signaling pathway with antisense to TGF- β ligand has been demonstrated to increase the tumorigenicity of quiescent colon cancer cells *in vivo* (38). In addition, globally decreased TGF- β activity may have deleterious effects in patients in terms of effects on cardiac disease, hypertension, or osteoporosis. Antisense approaches are also problematic in terms of the stability of current antisense constructs, the ability of antisense constructs to reach the target site, and the inability of this approach to produce sustained levels of effect. Therapy with neutralizing antibodies is limited by the ability to deliver

antibodies to solid tumors, as well as by the potential for formation of anti-idiotypic antibodies. Finally, therapy with IL-15 is limited, as IL-15 has been implicated in the pathogenesis of autoimmune diseases including rheumatoid arthritis (39).

On the other hand, blocking TGF- β signaling in only specific cells of the immune system (such as T cells) does not account for the potential effects of TGF- β on APCs and other components of the immune system that may be necessary for the local antitumor immune response. In addition, several of the approaches for specifically blocking signaling in T cells, though providing a compelling proof of principle, would be difficult to achieve in patients (i.e., transgenic expression of $T\beta RII\Delta cyto$ in host T cells). Expression of the dominant negative $T\beta RII\Delta cyto$ construct has the additional potential difficulties of generating immunity against the artificial $T\beta RII\Delta cyto$ protein, which may have long-term deleterious effects on treated patients, and blocking the TGF- β -signaling pathway at an early and likely common step in the pathway. As TGF- β has both positive and negative effects of T-cell function, this increases the possibility for nonspecific toxicity. Indeed, mice transgenic for human dominant-negative $T\beta RII\Delta cyto$ in T cells do develop a CD8+ lymphoproliferative disorder (21).

How might one overcome these potential difficulties? As TGF- β has numerous cellular effects, the first challenge is to more precisely define the TGF- β -signaling pathway, including specific pathways involved in immunosuppression, so that these pathways could be specifically targeted. Although Smad-mediated signaling remains the predominant TGF- β -signaling pathway, increasingly contributions by other signaling pathways (i.e., mitogen-activated and stress-activated protein kinase pathways) are being established and accepted. These pathways and other potential signaling pathways need to be explored, and the contributions of these specific pathways to the cellular effects of TGF- β established. Once the TGF- β -signaling pathway involved in immunosuppression has been more precisely defined, an attractive approach for targeting this pathway would be to utilize RNA-mediated interference (RNAi) technology. RNAi is an evolutionarily conserved, multistep process that produces small interfering RNA molecules (siRNA). These 21–23 nucleotide siRNA then result in specific degradation of homologous RNA, downregulating the mRNA and subsequently the resulting protein expression. RNAi is able to specifically and potently abrogate expression of targeted proteins in mammalian cells (40). Such an approach could generate tumor antigen-specific T-cell receptor-expressing T cells that specifically block the immunosuppressive actions of the TGF- β -signaling pathway through stable expression of RNAi constructs. This approach could initially be utilized to target $T\beta RII$ and the Smad3 transcription factor, as these two members of the pathway have both been shown to be necessary for T-cell function (22,41). This approach has the advantages that it could be applied to any component of the TGF- β -signaling pathway, including members of the pathway not yet elucidated, could target specific arms of the pathway (i.e., the immunosuppressive arm) and it could be expressed in various components of the immune system (T cells and APCs) to modulate the TGF- β -signaling pathway in numerous vaccine approaches.

The second challenge is to develop ways for specifically blocking the TGF- β -signaling pathway, not only in specific immune cells but also in the local tumor milieu. One potential method for achieving this goal would be to utilize antigen-specific T cells expanded ex vivo as agents for expressing products that block the TGF- β -signaling pathway in the local tumor environment. One agent that might be suitable for such an

approach is the soluble extracellular domain of T β RIII (sT β RIII). sT β RIII potently binds all three isoforms of TGF- β , and is able to specifically antagonize TGF- β signaling (42). This approach would have the following advantages: sT β RIII is a naturally occurring protein (as opposed to sT β RII or T β RII Δ cyto) so an immune response would not be generated; sT β RIII binds to all three TGF- β isoforms with high affinity (as opposed to T β RII, which does not bind TGF- β 2); sT β RIII would be produced locally by the T cells, thus minimizing systemic effects; and sT β RIII would affect not only the T cells secreting it but also APCs and any other immune cells in the local tumor milieu that are normally immunosuppressed. sT β RIII expressed in vivo has already been shown to successfully antagonize TGF- β signaling (43). An alternative approach would be to selectively block activation of TGF- β in the local tumor environment. As proteases are thought to have a potential role in this activation process, it is intriguing that treatment with protease inhibitors can downregulate tumor cell-produced TGF- β 's immunosuppressive effect in vitro (44). For such an approach to applied in vivo however, increased understanding of the mechanism of activation of TGF- β in vivo will be required.

6. SUMMARY/CONCLUSIONS

A large body of evidence supports a central role for the TGF- β -signaling pathway in mediated active tumor-induced immunosuppression. This immunosuppression directly interferes with the effectiveness of current immunotherapy strategies. Recent studies have demonstrated that targeting the TGF- β -signaling pathway may represent an attractive mechanism to enhance the effectiveness of immunotherapy in treating human cancers. Current approaches are limited by their nonspecific nature and potential for side effects. More precise delineation of the TGF- β -signaling pathway, particularly the specific pathways involved in mediating TGF- β 's effects on tumor-induced immunosuppression, will be necessary to more effectively target this pathway in a clinically relevant manner.

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II

GENERAL VACCINE AND IMMUNOTHERAPY STRATEGIES

8

Immune Adjuvants

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1. INTRODUCTION

The success of cancer immunotherapy is dependent on the discovery of tumor-associated antigens (TAAs) for vaccine targets, but equally important is the need for effective delivery strategies and potent adjuvants to induce an appropriate immune response. Although there is evidence that many cancer patients do mount an immune response against their tumor, the affinity and frequency of responding T cells appears to be low. Additionally, the “natural” immune response to some cancers is a Th helper 2 (Th2) response, whereas it appears that a Th1 inflammatory response, involving cytotoxic T lymphocytes (CTLs), may be necessary for effective antitumor immunity (1). Thus, the use of a potent Th1 adjuvant may provide sufficient exogenous danger signals to help tip the balance in favor of generating a protective immune response.

The most important properties of potent adjuvants for cancer vaccines are the ability to elicit cell-mediated immunity, specifically Th1 responses and CTLs; the ability to elicit T cells with a high affinity for the antigen in question; and the ability to elicit antibodies specific for tumor antigens expressed on the cell surface. These adjuvants will certainly be necessary for peptide or protein subunit vaccines, and will likely also enhance the efficacy of plasmid DNA and viral vector-encoded antigens (2,3).

Until recently the development of adjuvants has been a largely empirical process but scientific advances are more clearly defining their modes of action. This information can now be applied in the rational development of adjuvant formulations that address many of the unique immunoregulatory barriers associated with antitumor responses.

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2. THE ADJUVANTS

For the scope of this chapter, we will focus on only a few select adjuvants that have been in human clinical trials. For a more complete list of candidate adjuvants, readers are directed to the National Institute of Health (NIH) website for a Compendium of Adjuvants (<http://www.niaid.nih.gov/aidsvaccine/pdf/compendium.pdf>) (4). Historically, adjuvants were used to boost the immune response to infectious disease vaccine antigens and were generally evaluated by measuring antibody responses. Alum, an adjuvant effective at doing this with many whole-cell vaccines, was approved for clinical use in 1926 (5) and for the past 70 yr has remained the only adjuvant routinely used in commercial vaccines. Since then, advances in the field of vaccinology have provoked a renewed and intense interest in new and more potent adjuvants.

The complexity of most adjuvants coupled with the multifaceted nature of the induced immune response has made it exceedingly difficult to define the exact mechanism by which they exert their immune-stimulating activity. However, in a very general sense, adjuvants can be segregated into classes based on their modes of action (6). For example, some adjuvants act as depots shielding the antigen from rapid dispersal, degradation, and elimination. This depot effect allows for delayed release and prolonged exposure of the antigen to the immune system resulting in the promotion of a stronger response. The most notable adjuvants in this category include the aluminum salts and oil emulsions. A second major adjuvant class includes compounds known to trigger cytokine elaboration. The best-characterized adjuvants in this category are derived from bacterial products, or their synthetic analogs, and as such are ligands for Toll-like receptors (TLRs). MPLTM adjuvant and CpG oligonucleotides (ODNs) are prime examples of this class of adjuvants. Activation of TLRs through adjuvant binding results in stimulation of the innate immune response and secretion of a cascade of cytokines that ultimately stimulates the adaptive immune response. Other adjuvants function by enhancing presentation of antigen by antigen-presenting cells (APCs). This category includes compounds that encourage aggregate formation to target uptake by phagocytic APCs, as well as compounds that target antigens to the major histocompatibility complex (MHC) class I presentation pathway, which is critical for CTL induction. Saponins, such as QS-21 and GPI-0100, are adjuvants known for their ability to induce CTL responses with soluble protein antigens.

The classification of adjuvants by mode of action is obviously oversimplified and incomplete. Most adjuvants have multiple mechanisms of action and fall within several categories. Nonetheless, by matching the characteristic mode of action with what is required of a protective response, investigators can more logically select appropriate adjuvants. It also highlights the potential benefit of combining adjuvants with different characteristics into a single formulation to achieve a more robust response. A good example is the SBAS-2 adjuvant system from Glaxo-SmithKline, which unites the cytokine-stimulating capacity of MPL adjuvant with the CTL-inducing properties of QS-21 (7).

2.1. Bacterial Extracts in Cancer Vaccines

Over a century ago, a New York physician, William B. Coley, noted that some cancer patients experienced tumor regression following episodes of acute bacterial illness. Dr. Coley hypothesized that the two events were linked and treated an inoperable cancer patient with a viable bacterial culture to induce a “commotion in the blood.” Remarkably, the patient recovered from both the bacterial infection and the tumor. From 1900 to 1936,

Coley went on to successfully treat many tumor-bearing patients with heat-killed bacterial cultures known as Coley's Toxins (8,9). We now realize that Coley's Toxins contained at least two bacterial-derived molecules with potent immunostimulatory activity; lipid A and bacterial DNA (see "MPL Adjuvant and CpG").

During the period that Coley was treating cancer patients with his bacterial toxins, other scientists were documenting that antibody responses to experimental antigens were enhanced in animals infected with *Mycobacterium tuberculosis*. Several investigators went on to show that killed whole mycobacteria, as well as other Gram-positive and Gram-negative bacteria, enhanced the antibody response to exogenous antigens (reviewed in ref. 10). In the meantime, Arthur Johnson identified lipopolysaccharide (LPS) as the component of Gram-negative bacteria that had potent adjuvant properties (11).

This early work has led to the development of many of the modern immune adjuvants currently in clinical trials with cancer vaccines. Furthermore, the nonspecific approach used by Coley and others is still being investigated. For example, the therapeutic efficacy of bacille Calmette-Guérin (BCG) against superficial bladder cancer has been correlated with the secretion of Th1 cytokines and chemokines (reviewed in ref. 12). Additionally, an immunostimulant prepared from heat-killed *Mycobacterium vaccae*, SRL 172, has shown some benefit in human malignancies (13–16).

It is now known that host recognition of microbial components occurs through the recently described TLRs. This family of receptors and their associated intracellular messengers evolved to recognize and respond to conserved microbial components. The response manifests in an immediate activation of cells of the innate immune system resulting in the secretion of inflammatory cytokines, reactive oxygen intermediates, and defensins. In addition to providing immediate nonspecific protection, this inflammatory response induces and regulates the initiation of the antigen-specific adaptive immune responses, controlling dendritic cell (DC) maturation and differentiation of T helper (Th) cells (reviewed in ref. 17).

2.2. Oil-Based Adjuvants

Oil-based adjuvants are formulated as emulsions, which are dispersions of an antigen-containing aqueous phase with an oil phase. Water-in-oil (W/O) emulsions contain higher concentrations of oil, typically 30–80%, consist of water droplets immersed in an oil milieu, and are quite viscous. In contrast, oil-in-water (O/W) emulsions are composed of oil droplets immersed in an aqueous phase, and are predominantly aqueous in nature, with oil concentrations of only 2–10%. W/O emulsions are more apt to induce cellular immune responses, whereas O/W emulsions predominantly induce Th2 responses unless they are formulated with an additional immunomodulator. Both types of emulsions contain surfactants that, through their amphipathic nature, aid in the stabilization of the emulsion by interacting at the interface between the oil and water.

The adjuvant basis of emulsions is strongly related to their interaction with the antigen. They make the antigen particulate in nature through aggregate formation, and form depots allowing for slow release of the antigen. The degree of depot formation is dependent on the type and concentration of the oil used. Thicker, more viscous W/O emulsions cause significant depot formation and substantially prolong release of antigen. Additionally, emulsions induce inflammation at the injection site attracting macrophages to the area, which engulf the antigen and carry it to the draining lymph nodes (18).

Jules Freund was the first to develop oil-based adjuvants, and these formulations have been the principle adjuvants used in research laboratories for generations (19). Complete Freund's adjuvant (CFA) is a mineral oil W/O emulsion, which includes killed *M. tuberculosis* as an added immunostimulant. CFA is still considered one of the most potent adjuvants available, but the associated toxicity precludes its use in the clinic. Incomplete Freund's adjuvant (IFA) is a similar emulsion to CFA but lacking the mycobacterium component. IFA is demonstrably less toxic than CFA and has been utilized in modern vaccines, notably as an adjuvant for peptide-based strategies. These trials have reported the induction of immune responses in immunized cancer patients (20–23), but these responses have often been of low frequency (23) or have not been associated with a clinical improvement (20,22,24), suggesting the need for additional immunostimulants to improve the potency of the induced response. Indeed it has been shown that the coadministration of granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-2 (IL-2), or CpG with these formulations has improved immune responses and/or clinical outcome over those seen with IFA alone (21,25–27).

Mineral oils such as Drakeol, Bayol, or paraffin were traditionally used to formulate CFA and similar W/O emulsions (19). These complex mixtures of lipids with varying-length fatty acids are nonmetabolizable, leading to longer retention at the injection site and some local reactogenicity. Several groups continue to develop novel oil-based emulsions with the aim of retaining their potent adjuvant activity while reducing toxicity. Seppic's Montanide ISA 51 is a W/O emulsion analogous to IFA, but it is made with a more refined mineral oil. ISA 51 has been tested extensively in the clinics where it has demonstrated an acceptable level of toxicity (28). There are many ongoing peptide-based clinical trials in cancer patients using the ISA 51 adjuvant, alone or in combination with other immunostimulants (29). Published studies to date suggest the success of these types of formulations is modest and sporadic, similar to what has been seen with IFA (28,30).

Another W/O emulsion being tested in the clinics is Montanide ISA 720, also from Seppic, which is prepared with a metabolizable oil to reduce injection site reactivity associated with mineral oil (31–35). This adjuvant has also shown an acceptable toxicity profile for disease indications such as cancer.

O/W emulsions like those first developed by Ribi (36) provide another mechanism by which to reduce the toxicities associated with the more viscous and reactogenic W/O emulsions. Chiron Corporation's MF-59 adjuvant has an oil concentration of only 4.3% (w/v) requiring a microfluidization process to make the emulsion stable, yet fluid in nature. In addition to the low oil concentration, MF-59 is made with the metabolizable oil, squalene. MF-59 has been studied extensively in clinical trials with infectious disease vaccines (37,38) and is approved for use in Italy as part of an influenza vaccine (39).

2.3. MPL-Containing Adjuvants

Ribi et al. (36,40–43) carried out systematic studies on the antitumor activities of cell wall components from both mycobacteria and Gram-negative *Salmonella* species. The findings from these studies led to the development of two adjuvants currently in advanced clinical studies with cancer vaccines: Corixa Corporation's monophosphoryl lipid A (MPL™ adjuvant) and Enhanzyme™ immunostimulant.

LPS is the major component of Gram-negative bacterial cell walls. Evaluation of different LPS preparations for antitumor therapy indicated that the most effective

varieties were from the Re (rough) mutant strains of *Salmonella* (44) and subsequently MPL adjuvant was derived from the detoxified LPS of *Salmonella minnesota*, strain R595.

In general, LPS is composed of three distinct regions including the *O*-specific side chain containing repeat oligosaccharide units, a core structure composed of two distinct oligosaccharide regions, and the lipid A portion. The lipid A component is a diphosphate, β -1,6-linked D-glucosamine disaccharide with varying number of fatty acid tails and is predominantly responsible for the biological activity of endotoxin. It is an extremely potent biological stimulant that can induce toxic reactions in mammals at minute doses. Mild acid hydrolysis of purified *Salmonella* Re LPS breaks a covalent bond releasing the lipid A moiety from the rest of the molecule. One of the two phosphate groups is also selectively removed during this hydrolysis leaving the lipid A with a single phosphate group. A subsequent base hydrolysis selectively removes the fatty acid group from the 3 position yielding 3-*O*-deacyl monophosphoryl lipid A or MPL adjuvant. MPL adjuvant was found to have significantly reduced toxicity compared to the parent lipid A molecule yet still retained much of its inherent adjuvant activity (45,46). This important finding opened the door to the development of MPL as a vaccine adjuvant. Preliminary data now suggest that MPL, like its parent molecule, LPS, interacts with TLR-4 (unpublished data, 47).

MPL adjuvant has been used extensively in both preclinical and clinical settings. In mice, MPL adjuvant has been shown to enhance both humoral and cell-mediated immunity to a wide variety of different vaccine antigens (46). In these studies, MPL adjuvant mediated an overall increase in antibody titers as well as a qualitative shift toward isotypes capable of fixing complement and participating in antibody-dependent cell-mediated cytotoxicity (ADCC) reactions. Importantly, these antibody subclasses are considered more efficacious against tumor targets (48). MPL adjuvant promotes cell-mediated immunity with a predominantly Th1 phenotype and CTL (49, unpublished data). In clinical trials, MPL adjuvant has shown a similar capacity to generate a Th1 response characterized by Th cells, interferon- γ (IFN- γ), and complement-fixing antibodies (50–54). So effective is the ability of MPL adjuvant to bias the immune response toward a Th1 response that it is currently being used with allergy vaccines (54). MPL adjuvant has a safety profile similar to alum (55).

In murine tumor models, an O/W formulation of MPL adjuvant has generated promising data for the induction of CaMBr1, MUC-1, and GD-3-specific antibody responses (56,57), and IFN- γ responses to the carrier protein KLH (57). In human cancer vaccine trials, MPL adjuvant has, for the most part, been used in combination with other immunostimulants, such as QS-21 in SBAS-2 (58) and with cell wall skeleton (CWS) in Enhanzyn™ immunostimulant.

In order to isolate the powerful immunostimulatory properties of mycobacteria, bacterial fractions were methodically evaluated for antitumor activity using several tumor models (36,40–43). A highly active fraction was eventually isolated that contained the particulate CWS, a complex biological matrix consisting of three covalently linked macromolecules: peptidoglycan, arabinogalactan, and mycolic acids (59,60). This insoluble matrix is produced following the fracture of purified whole *Mycobacterium phlei* cells and then submitted to solvent extraction, protease, and nuclease treatment. These processes remove all but the most tightly bound lipids, carbohydrates, proteins, and

nucleic acids. CWS likely stimulates the innate immune response through interaction with TLR-2, as peptidoglycan and other mycobacterial wall components have been shown to do (61,62).

In animal models, CWS formulated with oil was able to mediate both the suppression and the regression of syngeneic tumors (36,40–43,59). Adding LPS to the CWS resulted in enhanced antitumor activity with faster response times, increased cure rates, and regression of well-established tumors (63,64). Importantly, animals that were cured of tumor burden were resistant to reestablishment of the tumors suggesting tumor-specific adaptive immune responses were induced.

Enhanzym™ immunostimulant (formerly known as Detox) is a potent adjuvant that was designed specifically for cancer immunotherapy. MPL adjuvant, CWS, and squalane oil are blended together as an O/W emulsion. This adjuvant system takes advantage of multiple mechanisms of action including the depot formation potential of the O/W emulsion and the cytokine-producing capacity of two distinct immunostimulants, which signal through TLR-4 and TLR-2. In both preclinical and clinical studies with Theratope®, the MUC-1 Sialyl-Tn (STn) carbohydrate vaccine from Biomira, Enhanzym immunostimulant has been shown to stimulate humoral and cell-mediated immunity with a strong potential for inducing DTH-type responses (reviewed in ref. 65). Enhanzym immunostimulant has been extensively evaluated in human clinical trials where thousands of doses of Theratope vaccine have been administered to ovarian and breast cancer patients (65). Comprehensive phase I and II safety data have been generated in over 400 patients, and the adjuvant/antigen combination is generally well tolerated with minimal toxicity. The results for a phase III randomized controlled study of Theratope vaccine for metastatic breast cancer will likely be reported during 2003.

Enhanzym immunostimulant has also been tested clinically as part of the melanoma lysate vaccine Melaccine. In a phase III trial, Melaccine prolonged relapse-free survival and overall survival in patients who expressed HLA-A2 and/or HLA-C3 (66). These findings suggest that the vaccine was able to induce a CD8+ T-cell response in these patients. In further studies, MUC-1 peptide delivered with Enhanzym immunostimulant induced specific CTL responses in 7 of 11 breast cancer patients (67), and Enhanzym immunostimulant codelivered with irradiated autologous tumor induced CTL in 10 of 35 vaccinated melanoma patients (68). In many studies a therapeutic effect has been correlated with the detection of tumor-specific CTL responses in immunized patients (67–70). It is likely that the adjuvant plays a significant role in induction of these responses and underlines the fact that potent combinations of immunostimulants are likely required for efficacy in cancer vaccines.

A new synthetic lipid A mimetic, RC-529, has been tested preclinically for adjuvant activity and was recently evaluated in a clinical trial. This molecule differs from natural lipid A disaccharide molecules in that it is an acylated monosaccharide (71), though it retains the ability to bind and signal through TLR-4 (unpublished data). In preclinical studies, RC-529 induced Th1 type of immune responses to a variety of vaccine antigens and promoted CTL induction (71). In human studies with a candidate hepatitis B vaccine, RC-529 promoted significantly greater seroprotection and higher mean titers than the nonadjuvanted vaccine. The safety profile was similar between the RC-529 adjuvanted and the nonadjuvanted vaccine (72).

2.4. CpG Oligonucleotides

While pursuing the tumor-regressing abilities of *Mycobacterium* components, Tonkunaga et al. (73,74) discovered the nucleic acid fraction was highly immunostimulatory. DNA isolated from BCG activated natural killer (NK) cells and stimulated IFN- γ secretion. These findings were confirmed by Krieg et al., who showed that bacterial DNA activated murine B cells resulting in proliferation and immunoglobulin secretion (75).

Bacterial DNA is a potent immunostimulator due to its ability to signal through TLR-9 (76). The differential immunostimulatory effect of bacterial, but not vertebrate DNA, is due to the presence of unmethylated CpG dinucleotides, which are preferentially methylated in mammalian DNA. Using synthetic oligonucleotides (ODNs), Krieg et al. (75) established that immunostimulatory activity is also affected by flanking nucleotide sequences. ODNs containing CpG motifs mimic bacterial DNA by inducing activation of APCs; in response to CpG, DCs express costimulatory molecules and secrete IFN- γ and Th1-promoting cytokines IL-12 and IL-18 (77–79). Numerous studies have highlighted the potential of CpG ODNs as vaccine adjuvants; they promote enhanced humoral and cellular responses with a strong Th1 bias (80–82), and have been shown to promote CTL responses (83,84).

CpG ODNs have been used as vaccine adjuvants to generate effective antitumor immunity in mouse models (26,85–88) and as monotherapeutics in the absence of exogenous antigen (89,90). Coley Pharmaceutical's CpG 7909 is currently in human monotherapy trials for melanoma, renal cell carcinoma, and non-Hodgkin's lymphoma. Results of these trials are eagerly awaited. Use of CpG 7909 as a cancer vaccine adjuvant is likely to be initiated in the next few years in collaboration with GlaxoSmithKline and with The Ludwig Institute.

2.5. Saponins

The saponins are a heterogeneous family of surfactant molecules generally isolated from plants and have long been appreciated for their adjuvant activity. These complex glycosidic molecules contain a quillaic acid backbone linked to various sugar moieties (91). The amphipathic nature of the saponins is due to the presence of both hydrophilic sugars and hydrophobic triterpene core. Dalsgaard et al. (92) originally evaluated the adjuvant activity of a number of saponins and concluded the strongest immunostimulants were from extracts of the South American soap tree, *Quillaja saponaria*. Although the crude extracts were potent adjuvants, they induced unacceptable levels of toxicity, partly due to hemolysis. Dalsgaard partially purified the crude extracts using dialysis and chromatography resulting in a fraction called Quil-A, which had reduced toxicity but retained much of its adjuvant activity. The Quil-A fraction has been used extensively in research and as an adjuvant for veterinary vaccines but is considered too toxic for human use.

Analysis of Quil-A by high-performance liquid chromatography (HPLC) revealed a heterogeneous mixture of at least 24 related saponin species. It was later determined that not all the compounds were equally effective adjuvants but had varying degrees of biological activity. Kensil et al. (93) discovered the four major fractions all possessed strong adjuvant activity but exhibited widely different levels of toxicity. The most predominant fraction, QS-18, was extremely toxic in mice, but another fraction, QS-21, had acceptable reactogenicity levels while maintaining potent adjuvant activity. In mice QS-21 has been

shown to enhance both humoral and cell-mediated responses to a wide variety of antigens. The antibody responses are balanced with high levels of both IgG1 and IgG2a isotypes being produced, and murine studies have demonstrated the capacity of QS-21 to activate CTL responses (94). The mode of action of QS-21 has not been fully defined, but an aldehyde group at position 4 of the triterpene moiety and a glycosidically linked fatty acid group have both been implicated as important contributors to adjuvant activity (95). In one study, the deacylation of QS-21 was shown to severely compromise its ability to induce IgG2a antibodies and CTL to vaccine antigens (96).

QS-21 was the first saponin adjuvant to be tested in human clinical trials and demonstrated an acceptable safety profile (97). QS-21 has been tested extensively in preclinical mouse studies and in human trials with carbohydrate-KLH conjugate vaccines, where it has shown success in promoting IgG induction to these T-independent antigens, bypassing the need for specific T-cell help (98–101). However, induction of T-cell responses with peptide vaccines has generated mixed results (102–104).

Together with MPL adjuvant, QS-21 is a component of Glaxo-SmithKline's SBAS-2 adjuvant tested in the clinic with MAGE-3 in patients with metastatic cancer. Some clinical response was reported in 5 of 33 melanoma patients enrolled in this trial (58), though the evaluation of immune responses in these patients is yet to be published.

GPI-0100 is a semisynthetic derivative of the saponins from *Q. saponaria*. The ester-linked glycosidic acyl chains unique to quillaja saponins are readily hydrolyzed at physiologic pH and their loss dramatically reduces the adjuvant activity of these compounds. The toxicity related to saponins is also lost, implicating the fatty acid moiety as an intrinsic contributor to toxicity. Marciani et al. (105) designed saponin derivatives in which the unstable ester-linked acyl side chains were replaced with more stable amide linked acyl groups. In the case of GPI-0100, the replacement of the fatty acid was accomplished by the attachment of dodecylamine to the glucuronic acid moiety. This created a more stable compound with dramatically reduced toxicity, but maintained adjuvant capacity.

In mice, GPI-0100 adjuvant enhances both humoral and cellular immune responses. Notably, when used with conjugate vaccines in mice, GPI-0100 and QS-21, alone or in combination with other immunostimulants, induced the highest antibody and T-cell responses of a range of adjuvants tested (106,107). A phase I study is underway in patients with prostate cancer testing a dose escalation of GPI-0100 with a glycosylated MUC-2-Globo H-KLH conjugate vaccine.

The glycosides of Quil A can also be formulated with cholesterol, phospholipids, and antigens to form immune-stimulating complexes (ISCOMs) as first described by Morein (108). The incorporation of Quil A into the ISCOM matrix reduces the intrinsic toxicity of the saponins. ISCOMs have a cage-like structure, which is created by the micellar formation of Quil A and cholesterol (109). The addition of phospholipids seems to be required for the incorporation of some protein antigens. However, most viral membrane proteins with their hydrophobic transmembrane regions readily incorporate into the ISCOM structure. In murine studies, ISCOMs enhance both humoral and cellular immune responses. The adjuvant activity is dependent on the Quil A component but may also be a result of a depot effect allowing slower release of the antigen from the injection site (109). It has been speculated that the ability of ISCOMs to mediate CTL responses is because of their ability to penetrate APC membranes and deliver antigen to the endog-

enous class I presentation pathway. Due to their compatibility with viral membrane proteins, ISCOMs are an attractive adjuvant for vaccines against oncogenic viruses.

2.6. Cytokines

Rather than using more traditional adjuvants to induce a cascade of cytokines, one can administer individual cytokines or their genes in combination with TAAs as vaccines to augment antitumor immune responses. This approach has shown some promise in the clinic, but has also encountered some significant problems with systemic toxicities (110–112). The most common cytokines utilized in mouse experiments and human trials are GM-CSF, IL-2, and IL-12. GM-CSF is a growth factor that has multiple effects on many cell types, including macrophages and DCs. Included in these effects is increased production and differentiation of DCs, enhancement of antigen presentation by APCs, and induction of cytokines, including IL-12, by macrophages. GM-CSF has shown efficacy as an adjuvant for tumor vaccines in animal models (113,114) and in a rat model, it was as effective as CFA for the generation of a DTH response to rat neu (115). Moreover, GM-CSF has been found to be generally well tolerated and has shown promise as a vaccine adjuvant in people when administered with peptides. The combination of TAA peptides plus GM-CSF has been shown to be efficient for the generation of CD4+ (116,117) and CD8+ T-cell responses that correlated with clinical response in immunized patients (118,119). One study directly compared three adjuvants and concluded that GM-CSF was superior to IFA for its ability to augment the frequency of tyrosine peptide-specific T cells in melanoma patients (104). Alternatively, irradiated autologous tumor cells, engineered to express GM-CSF, have shown activity in animal models (113), and enhanced T-cell responses in patients, including infiltrates at the metastatic tumor sites (120–122). Notably, the combination of GM-CSF with CpG adjuvant or with IL-12 enhances anti-tumor immune response in mouse tumor models (88,123). Adjuvant combinations such as this have great potential for future clinical application with active specific immunotherapy of cancer.

IL-2 is a T-cell growth factor that has been used to induce antitumor responses in preclinical models and in patients for many years. However, at the high doses often required for these studies, there have been cases of extreme toxicities and even death (110,124,125). It is likely that this cytokine's antitumor effect is mediated by its ability to stimulate existing CD4+ and CD8+ T cells, thus the combination of IL-2 and specific vaccines was a logical extension of these early studies. IL-2 plus peptide-mediated cancer regression in 42% of patients with metastatic melanoma (21). Follow-up studies suggested that administration of this cytokine resulted in sequestering of specific T cells at the tumor site, since a clinical benefit was correlated with a decrease in circulating peptide-specific CD8+ T cells (27). IL-2 is included as an adjuvant in many peptide-based cancer vaccine trials currently open for enrollment (29).

IL-12 augments Th1 and CTL responses. In preclinical models IL-12 has a dramatic antitumor effect (111) due to induction of a cascade of proinflammatory cytokines and direct effects on the tumor and angiogenesis (126). IL-12 augmented T-cell responses to a multipeptide vaccine in the majority of immunized patients with resected metastatic melanoma (127) and enhanced CD8+ T-cell responses when it was coadministered with MAGE-3 or MelanA peptide-pulsed peripheral blood mononuclear cells (PBMCs) (128). However, repeated administration of IL-12 over time has been documented to result in

a reduction in clinical and immunological benefits, possibly through IL-10 induction (129).

3. DISCUSSION

The exploration of improved vaccination strategies, including adjuvants, for delivering TAAs and achieving the appropriate tumor-specific immune responses has become a major priority as the number and types of epitopes has increased. For example, the induction of CD8+ CTL responses with soluble proteins has traditionally been challenging, and these types of vaccines in particular will require a means to deliver antigen into the class I pathway and a potent adjuvant formulation to provide exogenous signals for DC maturation *in vivo*.

Review of the data generated on the use of immune adjuvants in clinical trials with cancer vaccines highlights the need for more comparative studies in which the adjuvant itself is the only variable. Only with these kinds of systematic studies can one begin to make general conclusions as to which adjuvants work best with peptide-, protein-, carbohydrate-, and gene-based approaches.

Kim et al. (57,107) performed two extensive comparisons of adjuvants, and combinations thereof, in a preclinical model with their MUC-1 and GD-3 KLH conjugate vaccines. Saponins were superior for induction of antibodies against the carbohydrate antigens. In humans, Schaed et al. (104) found that both QS-21 and GM-CSF were superior to IFA for the generation of T-cell responses to an HLA-A0201-restricted tyrosinase peptide. Slingluff et al. (28) compared ISA 51 with QS-21 for the ability to generate responses to a gp100 peptide in 22 patients with resected melanoma. However, there was no significant difference between responses generated with either adjuvant, and so the study was inconclusive in this regard. Finally, IL-2, GM-CSF, and IL-12 were compared as adjuvants for a melanoma peptide, and in this case, IL-2 was found to be associated with the best clinical response (27). In general, one can say that GM-CSF, QS-21, and Enhanzym immunostimulant have all shown some notable successes in the clinic, but it is certain that further improvements are needed. The forthcoming data on the use of CpG ODNs in cancer patients may provide a basis for future directions.

Recent advances in understanding how adjuvants work will allow for the design and manufacture of synthetic molecules with enhanced activities and, in some cases, decreased toxicities. Synergistic combinations of two or more immunomodulators can target multiple TLR receptors or other stimulatory pathways. Such combinations will likely be required for cancer vaccines where unique immunoregulatory hurdles to “self” antigens must be overcome. Such potent adjuvant formulations may induce a greater degree of injection site reactogenicity than would be acceptable with prophylactic vaccines, but this should not discourage us from their use in the clinic where the benefit may far outweigh local toxicity issues.

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9

Heterologous Prime-Boost Vaccination in Tumor Immunotherapy

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1. INTRODUCTION

Most of the vaccine strategies under development use multiple immunizations with the same agent such as a viral vector encoding a tumor antigen. More recently, the concept of heterologous prime-boost immunization has been tested in animal models. This strategy involves the sequential administration of *different* delivery vectors encoding the *same* recombinant antigen (1). Initially demonstrated in animal studies of infectious diseases, such as malaria (1,2) and AIDS (3), prime-boost technology is now being developed for use in tumor patients (4).

The principle of prime-boost technology is to force the immune system to focus its response on a specific recombinant antigen by avoiding the preferential expansion of vector-specific cytotoxic T lymphocytes (CTLs) that occurs after sequential administration of the same delivery vector in homologous prime-boost regimens. Administration of the first immunogen primes CTLs specific for the recombinant antigen, and also for the delivery vector. If an unrelated second vector is administered during the “boost” phase, the immune system is faced with a wealth of new vector antigens. Because the vector also encodes the recombinant antigen, for which primed cells already exist, the immune system raises a massive memory response, expanding previously primed CTLs, which are specific for the recombinant antigen only (Fig. 1; 4).

Examples of recombinant vectors used in heterologous prime-boost vaccine regimens in animal models of cancer and infectious diseases are: plasmid DNA, members of the

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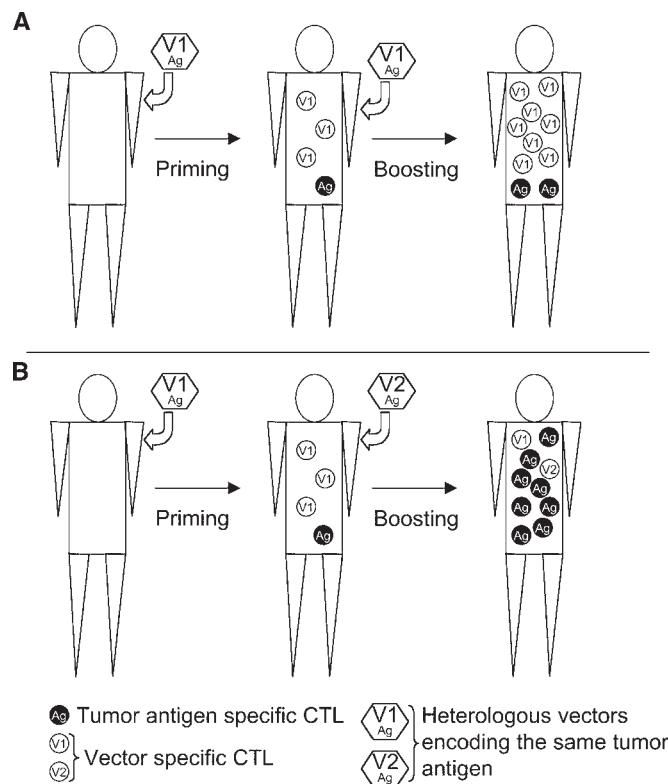


Fig. 1. (A) Homologous prime/boost. Priming with a recombinant vector (V1) and boosting with the same vector leads to dominant amplification of CTL responses specific for vector antigens. (B) Heterologous prime/boost. Priming with a recombinant vector (V1) generates memory CTL specific for recombinant antigen (Ag), which are effectively boosted by a second recombinant vector (V2) encoding the same recombinant antigen.

Poxviruses including vaccinia virus, modified vaccinia Ankara (MVA), canarypox (ALVAC) and fowlpox, as well as Semliki Forest virus (SFV), influenza virus, and adenovirus. Many different combinations of these delivery vectors have been used for prime-boost protocols in animal models in infectious diseases and cancer. In addition, priming with a viral vector may be followed by boosting with peptide, protein, or antigen-loaded dendritic cells (DCs) (5). The safety of many of these delivery vectors has been confirmed in clinical trials. However, very few clinical trials of prime-boost protocols in the field of oncology have been published to date (5,6).

2. ADVANTAGES OF HETEROLOGOUS PRIME-BOOST IMMUNIZATIONS

Heterologous prime-boost immunizations have the following advantages over repeated immunizations with the same vector. First, vector specific immune responses are not boosted (*see* Fig. 1). Second, the best immunostimulatory properties of each strategy may be combined. Third, any toxicities of a vector are limited by the lower frequency with which any one vector (particularly those that induce significant inflammation) need be administered.

Regarding vector-specific immunity, antivector immunity might neutralize the vector before it has the chance to infect the recipients' cells, thereby preventing the production of its encoded antigens. The presence of neutralizing antibody has been shown to reduce the immunogenicity of vaccines in animal models. In a murine system, neutralizing antibodies specific for the hemagglutinin (HA) of influenza virus were shown to inhibit a boost of the immunodominant response against influenza expressing the same HA. When influenza virus with a non-cross-reactive HA was used for boosting, responses specific for the immunodominant NP-epitope were boosted 10-fold (7,8). Another study demonstrated that neutralizing maternal antibodies in newborn humans interfere with the effectiveness of measles vaccination (9). A second mechanism for interference from preexistent immunity to the vector is immunodominance: CTL specific for the vector can compete with CTL specific for the recombinant antigen around the antigen-presenting cell (APC). It is therefore important to monitor patients not only for antitumor immunity but also for vector-specific T- and B-cell responses.

For future vaccine design, it might be preferable to utilize recombinant viral vectors that usually do not infect humans, minimizing the chance of preexisting antivector immunity in the patient. An example of such a vector, which has recently been shown to be effective in prime-boost vaccinations, is replication-incompetent SFV, a murine pathogen that encodes a minimal amount of vector antigens, yet is highly immunogenic. Alternatively, choosing the right vaccine vectors might involve the screening of human populations for preexisting responses against vector antigens.

Regarding combining the ideal immunostimulatory properties of each vector, it is clear that different immunization platforms have different abilities to stimulate certain aspects of the immune response. For example, plasmid DNA results in persistent, but low levels of antigen expression. This may result in activation of small numbers of T cells. Also, plasmid DNA contains CpG dinucleotide motifs that activate innate immune responses. Viral vectors may cause high levels of antigen expression and T-cell activation, which in the absence of priming against the recombinant antigen would result in overwhelming antivector immune responses. If high-affinity T cells have been activated by a priming with a plasmid vaccine, then the high-affinity T cells can be expanded by the viral vector (11).

Regarding tolerability, using immunogens that do not have significant local skin toxicity as part of the immunization strategy will greatly increase patient tolerability. Although vaccinia vectors can cause substantial local reactivity (especially in those who have not been previously immunized against vaccinia), avipox as nonreplicating viruses, cause less local toxicity and DNA vectors cause none by themselves.

3. ROLE OF PRIME-BOOST FOR POLY-EPITOPE VACCINES

Polyvalent vaccines are being developed to induce broad CTL responses against multiple tumor antigens and epitopes restricted by more than one major histocompatibility complex (MHC) class I molecule. Poly-epitope vaccines aiming to induce such polyvalent responses have been tested in combination with the heterologous prime-boost approach. Instead of engineering single epitopes or antigens into heterologous vectors, poly-epitope and/or poly-protein expressing constructs have been used to drive the expansion of several CTL specificities. It has been shown that a “string-of-beads” assembly of known epitopes into artificial new poly-epitope genes can lead to simultaneous priming

of CTLs with a range of specificities (12). However, we and others have shown that, during the boosting step, competition of CTLs around the APCs is a major factor in reducing the breadth of a primed response (4,13). By simultaneously monitoring CTLs of several different specificities ex vivo we have shown that after boosting a poly-epitope-primed response, CTLs of a single specificity expand, leaving those of other specificities behind (Fig. 2; 4). The phenomenon observed is termed immundomination (4). Recently, in this setting, a number of groups have investigated the underlying mechanism of immundomination (4,13–15). It has been shown that competition of CTLs around APCs (in particular DCs) can cause the inhibition of subdominant CTLs by other CTLs specific for the dominant determinant. It is possible to eliminate the effect of immundomination either by increasing the number of APCs displaying a set of epitopes, or by expressing single epitopes/antigens on separate APCs. Simultaneous expansion of dominant and subdominant CTLs specific for a poly-epitope melanoma vaccine has been achieved by separating the epitopes on different APCs during the boosting phase of the vaccination (4).

4. ORDERING OF PRIME AND BOOST IMMUNIZATIONS

Although murine models have demonstrated the efficacy of prime-boost immunizations against tumor antigens, the best combination of platforms for immunization and the best order for delivery are still being evaluated. Schлом's group at the National Cancer Institute has demonstrated in mice transgenic for the human CEA gene that primary immunization with a vaccinia vector encoding CEA (rV-CEA) and costimulatory molecules followed by boosts with a fowlpox vaccine encoding the same proteins could generate potent CEA-specific T-cell responses that reduced tumor progression and increased survival (16). When used with DNA vaccines, it appears that attenuated vaccinia vectors should be administered as a boost (at least in infectious-disease models) (2). Similarly in a murine model of pre-B-cell leukemia, priming with a DNA plasmid vaccine followed by boosting with a vaccinia-based vaccine (both expressing a peptide derived from the tumor-specific immunoglobulin) conferred the greatest protection against tumor challenge (17). In a murine model of hepatocellular carcinoma, plasmid DNA-encoding murine α -fetoprotein (AFP) followed by boosting with an AFP-expressing adenoviral vector protected against tumor challenge as effectively as DC immunizations (18). Finally, DCs infected with viral vectors or loaded with tumor antigens can also be used in prime-boost strategies. DCs transduced with adenovirus encoding melanoma antigens followed by melanoma peptide-pulsed DCs induce the most potent tumor-specific CTLs (19). Because DCs are infected with the vector, it is possible that antivector immune responses are not as significant. Thus, immunization strategies using vector-modified DCs may overcome some of the limitations imposed by immune responses against viral vectors.

5. CONCLUSIONS

Heterologous prime-boost strategies increase the immune response relative to repeated immunizations with the same immunizing platform. The large number of immunization strategies under development will ultimately need to be tested to determine which combinations and in which order result in the greatest immune responses.

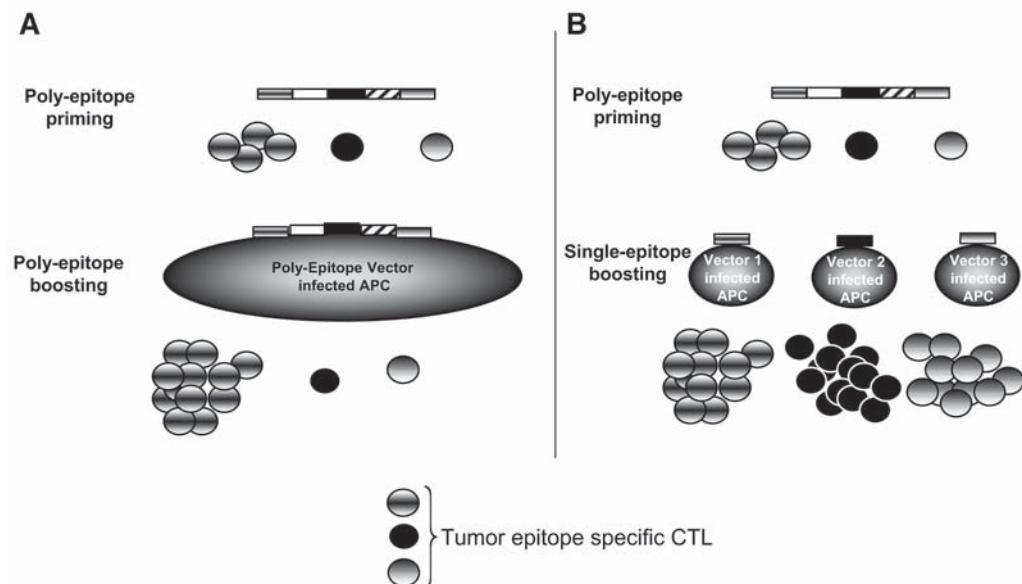


Fig. 2. (A) Poly-epitope priming followed by poly-epitope boosting skews the CTL response toward a single CTL specificity. (B) Poly-epitope priming followed by boosting with separate vectors encoding single epitopes allows the efficient expansion of several tumor-specific CTL specificities simultaneously.

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- INTRODUCTION
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1. INTRODUCTION

The identification of specific tumor antigens has significantly advanced the field of tumor immunology, in particular, the development of cancer vaccines. Improved understanding of the molecular basis of antigen recognition has resulted in the development of rationally designed tumor antigen-specific vaccines based on motifs predicted to bind to human class I or class II major histocompatibility complex (MHC) molecules. The use of synthetic peptides in cancer vaccines offers practical advantages such as relative ease of construction and production, chemical stability, and a lack of infectious or oncogenic potential (1). Peptides may also allow better manipulation of the immune response through the use of epitopes designed for stimulating particular subsets of T cells. Most importantly, peptide vaccines appear to be effective in generating immune responses to self-proteins (2). Theoretically, immunization to foreign proteins normally elicits immunity to only a subset of potential epitopes, dominant epitopes, whereas other potentially immunogenic epitopes, subdominant epitopes, are ignored. There is evidence that T cells are tolerant to the dominant epitopes of self-proteins, but may respond to the subdominant epitopes (3,4). As many newly defined tumor antigens are self-proteins, peptide immunization may play a key role in the ability to elicit an immune response to tumor antigens.

Peptide-based vaccines have advanced from preclinical to human clinical studies over the last decade and several important issues have been elucidated during this clinical

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progression. First, investigators have developed powerful paradigms to choose peptide(s) for immunization and established the preclinical evaluation needed to develop a peptide-based tumor vaccine. Secondly, the importance of class II peptides in active immunization is being increasingly defined. The generation of an endogenous CD4+ T-cell response will improve the magnitude and maintenance of CD8+ immunity generated with class I binding epitopes. Finally, methods have been developed to modify peptides to improve the immunogenicity of epitope-specific vaccines. Tumor antigen-specific peptide-based vaccines have been an excellent model to test active immunization in cancer patients over the last several years. Current work seeks to optimize vaccine formulations based on lessons learned in early clinical trials.

2. CLASS I PEPTIDES

CD8+ cytotoxic T cells (CTLs) can kill cells expressing an antigen. The action of CD8+ T cells requires recognition of antigenic proteins that have been processed and presented as peptide fragments in the context of class I MHC.

2.1. *Characteristics of Class I Peptides*

Epitopes recognized by CD8+ T cells are, in general, peptides of 8–10 amino acids in length anchored at each end within the major groove of MHC class I molecules. As a consequence, class I molecules exhibit more amino acid sequence specificity based on these anchoring residues, than class II molecules, which may be more promiscuous in the binding of peptides. Most investigators have focused on defining class I epitopes for human leukocyte antigen (HLA)-A2 molecules (Table 1). Most likely the interest in HLA-A2 is fueled by the increased prevalence of this allele in many human populations. However, as early studies of peptide-based vaccines have shown that cancer patients can be immunized against a variety of tumor antigens, more studies are focusing on HLA-A alleles other than A2 as well as HLA-B motifs. The frequency of common HLA-B alleles is shown in Table 2. A potential problem in the development of CTL epitope-based vaccines is the large degree of MHC polymorphism and the need for knowledge of HLA restrictions in the population to be vaccinated. However, it is now known that that HLA class I molecules can be divided into several families or supertypes based on similar peptide-binding repertoires (5). For example, the A2 supertype consists of at least eight related molecules and of these, the most frequently observed are HLA-A*0201, A*0202, A*0203, A*0206, and A*6802. In addition, the A2 supertype is expressed in all major ethnicities; 39–46% range of most common populations. Many peptides that bind A*0201 also exhibit degenerate binding, i.e., binding to multiple alleles; thus, an A2 supertype multipeptope vaccine could be designed to provide broad population coverage (5). Recent investigations have demonstrated that peptides that bind HLA-A0201 with high affinity will cross-react with other A2 family alleles (6). Studies of in vitro binding of peptides to other HLA-A molecules demonstrated that over 70% of peptides that bound HLA-A0201 with high affinity bound at least two other members of the A2 superfamily. Although class I motifs have been assumed to be very specific and restrictive, studies such as those described above suggest large overlaps in specificity can be found. As newer clinical trials translate these in vitro observations of the broadening of class I activity in vivo, it may be that class I peptide vaccines will be less restrictive in use than what had been previously assumed.

Table 1
Genotypic Frequency of Common HLA-A Antigens
in the North American Population

<i>Population</i>	<i>%HLA-A1</i>	<i>%HLA-A2</i>	<i>%HLA-A3</i>
Caucasian	15	29	13
African American	6	19	8
Asian/Pacific Islander	4	25	3
Hispanic	7	28	8
Native American	12	29	11

From refs. 75 and 76.

Table 2
Genotypic Frequency of Common HLA-B Antigens
in the North American Population

<i>Population</i>	<i>%HLA-B7</i>	<i>%HLA-B8</i>	<i>%HLA-B44</i>
Caucasian	12	9	13
African American	11	4	7
Asian/Pacific Islander	4	1	6
Hispanic	5	4	10
Native American	11	9	12

From refs. 75 and 76.

2.2. Identification of Class I Peptides

Initial studies of CTL epitopes were based on the elution of naturally processed peptides from class I MHC molecules on tumor cells. The identification of sequence motifs from eluted peptides, as well as crystallographic data of peptide binding to specific class I molecules has allowed predicted binding of specific peptides to class I molecules. Elution of peptides from MHC expressed on tumor cells is labor intensive and requires much technical skill as well as the instrumentation to sequence the eluted peptides. For this reason, several algorithms have been developed to predict binding motifs for the most common class I and class II MHC molecules. In general, once potential CTL epitopes have been identified by sequence analysis, some assessment as to their utility as MHC class I antigens needs to be made. Several systems have been developed to assess class I binding such as binding to empty MHC molecules (7) or the generation of CTLs by *in vitro* priming of peripheral blood lymphocytes (8). Many groups developed the paradigm of predicting a peptide sequence, assessing the ability of the peptide to bind to a particular MHC molecule *in vitro*, and then attempting to prime human T cells *in vitro* against the peptides that bound class I most vigorously. More recent investigations suggest that computer algorithms designed for predicting class I binding are excellent predictors of peptides that are natural epitopes of tumor antigens and that MHC-binding assays do not need to be used prior to assessing peptides against human lymphocytes *in vitro* (9). Lu

and colleagues used two computer-based prediction algorithms that are readily available on the Internet; syfpeithi.bmi-heidelberg.com and bimas.dcrt.nih.gov/molbio/hla_bind/hla_motif_search_info.html. The algorithms were able to identify three candidate peptides of carcinoembryonic antigen (CEA) specific for HLA-B7. After constructing the peptides based simply on motif identification, without testing for binding to HLA-B7 in vitro, investigators demonstrated one of the peptides generated CTLs capable of lysing HLA-matched CEA-expressing tumors. Similar strategies have been extremely useful for identifying class I peptide epitopes from a variety of tumor antigens. For example, Rongcun and colleagues identified four HER-2/neu-specific HLA-A2.1-restricted CTL epitopes; HER2(9₃₆₉), HER2(9₄₃₅), and HER2(9₆₈₉), HER2(9₆₆₅), which were able to elicit CTLs that specifically killed peptide-sensitized target cells, and most, importantly, a HER-2/neu-transfected cell line and autologous tumor cells. In addition, CTL clones specific for HER2(9₃₆₉), HER2(9₄₃₅), and HER2(9₆₈₉) epitopes were isolated from tumor-specific CTL lines, further demonstrating the immunogenicity of these epitopes (10).

Once a class I specific peptide has been identified, one must determine whether that peptide can be used to generate T cells capable of responding to and lysing HLA-matched tumor that expresses the antigen of interest. These studies may be performed by using peptide to sensitize T cells in vitro. However, in vitro propagation of human lymphocytes is labor intensive and operator dependent. In addition, establishing T-cell lines using lymphocytes derived from patients with cancer may be made more difficult due to potential inherent defects in the T-cell response in tumor-bearing hosts. For these reasons, another approach has been developed to rapidly evaluate the utility of predicted class I epitopes, active immunization of HLA-A2 transgenic mice. Vaccinating mice with class I peptides allows a more robust immune response to be generated to a particular tumor antigen than the response found endogenously in cancer-bearing patients. Moreover, initiating in vitro culture with a robust T-cell precursor frequency allows a more rapid expansion and evaluation of tumor-specific T cells. Screening epitopes using an HLA-A2 transgenic mouse has become a powerful tool in the rapid evaluation of class I peptides for inclusion in cancer vaccines and has been used successfully in assessing epitopes of HER-2/neu (11) and p53 (12). Technical advances in both the rapid prediction of class I motifs as well as in vitro and in vivo screening of the ability of peptide-specific T cells to respond to intact tumor cells has resulted in numerous class I epitopes being identified for a variety of antigens associated with diseases such as melanoma, breast, ovarian, lung, and colon cancer.

2.3. Patients With Cancer Can Be Immunized With Class I Peptide-Based Vaccines

Clinical trials performed vaccinating cancer patients with class I binding peptides derived from a variety of tumor antigens have demonstrated that patients can be immunized to boost immunity to self-tumor antigens. Peptide-based vaccines offer an excellent model for assessing the ability to immunize by measuring immunity generated with assays that can specifically measure class I T-cell responses. Lee et al. (13) used MHC tetramer flow cytometry to evaluate in vivo response to vaccination with gp100_{209–217} and tyrosinase_{368–376} peptides in 44 HLA-A2 patients with advanced-stage melanoma. Tetramer assays performed before, during, and following the period of vaccination demonstrated appearance of gp100-specific T cells as early as 4 wk, i.e., after two vaccinations. At 18 wk at least 20% of CD8+ T cells were specific for gp100_{209–217} in four of six

patients. Overall, 37 of 42 patients developed an immune response to gp100_{209–217} following vaccination as measured by MHC tetramer assay. Conversely, only minimal increases were seen in tyrosinase_{368–376}-specific T cells on tetramer assay following vaccination. Monitoring immunity to specific peptides will also allow information to be collected concerning the evolution of the immune response after active immunization. Speiser and colleagues (14) characterized Melan-A-specific cells in melanoma patients as compared to volunteer controls. The cell-surface receptors HLA-DR, CD45RA, CCR7, CD28, and 2B4 were assessed to evaluate differential expression in response to in vitro antigen stimulation. In volunteer donors, Melan-A-stimulated T cells manifested a phenotype characteristic of naïve cells. In melanoma patients, Melan-A-stimulated T cells expressed a partially activated phenotype and Melan-A-stimulated tumor-infiltrating lymphocytes expressed the phenotype of an effector cell. Following vaccination with Melan-A peptides, patients with advanced-stage melanoma demonstrated increased frequencies of Melan-A-specific T cells, and transition of cells from a naïve state to a more activated state via upregulation of HLA-DR and 2B4 and downregulation of CD45RA and CCR7. ELIspot assays of these cells were predominantly negative, consistent with incomplete transformation of T cells from naïve to effector phenotypes.

Most importantly, these early studies of class I specific peptide-based vaccines are beginning to show evidence of clinical responses. In a recent clinical trial performed by Scheibenbogen et al. (15), two patients with stage IV melanoma received immunization with tyrosinase peptides (tyrosinase_{368–376} in Patient 1, and tyrosinase_{206–214} in Patient 2). Patient 1 had experienced nine relapses over the 3-yr period before vaccination, had a nodal recurrence occurred following the sixth vaccination, but then remained relapse free for 30 mo. Patient 2 had experienced 12 relapses in the 3 yr prior to vaccination, then following vaccination remained free of disease for 27 mo. At 27 mo a visceral recurrence was resected, and Patient 2 remained disease-free for an additional 8 mo at the time of the report. This study highlights the prolonged relapse-free survival peptide-specific tumor vaccines may be able to induce in patients with advanced-stage malignancy.

3. CLASS II PEPTIDES

CD4+ T helper (Th) cells have the potential to augment the antigen-specific immune response via cytokine secretion. Th1 subsets of CD4+ T cells secrete cytokines such as interleukin-2 (IL-2) and interferon-γ (IFN-γ) that will stimulate the proliferation and activity of CTLs. Th2 subsets of CD4+ T cells secrete cytokines such as interleukin (IL)-4, 5, 6, and 10 that will result in more effective antibody formation. The action of CD4+ T cells requires recognition of antigenic proteins that have been processed and presented as peptide fragments in the context of class II MHC.

3.1. Characteristics of Class II Peptides

Unlike MHC class I molecules, which typically bind peptides of 8–10 amino acids in length, MHC class II molecules are generally believed to be more permissive in the length and exact amino acid sequence of bound peptide (16). For example, in studies defining the Th epitopes of tetanus toxoid, panels of overlapping peptides were screened for their ability to stimulate T-cell proliferation in lymphocytes derived from tetanus-immune subjects. Peptides of greater than 12 amino acids and less than 31 amino acids and typically in the range of 14–16 amino acids were found to be most efficient in defining

Table 3
Genotypic Frequency of Common HLA-DR Antigens
in the North American Population

<i>Population</i>	<i>%HLA-DR1</i>	<i>%HLA-DR3</i>	<i>%HLA-DR4</i>
Caucasian	10	11	17
African American	7	13	6
Asian/Pacific Islander	3	5	15
Hispanic	8	8	21
Native American	8	10	20

From refs. 75 and 76.

recognized epitopes (17). Moreover, Th peptides can be “universal” or promiscuous and bind to multiple alleles. Promiscuous class II helper epitopes have been identified for the HER-2/neu (18) and NY-ESO (19) tumor antigens.

3.2. Identification of Class II Peptides

Both CD8+ and CD4+ T cells play an important role in the generation of a potential antitumor immune response. CD4+ Th cells initiate and maintain CD8+ T-cell responses (20,21). Peptide-based cancer vaccines must consider the important and potentially critical role of CD4+ T cells. The identification of Th epitopes has traditionally been done either by eluting peptides bound to specific MHC class II molecules or by screening panels of overlapping peptides derived from the antigen being studied. The first method, eluting and sequencing peptides bound to specific MHC class II molecules from antigen-presenting cells (APCs), has been used to identify MHC class II epitopes associated with insulin-dependent diabetes (22), multiple sclerosis, and rheumatoid arthritis (23), as well as from the melanoma tumor antigens tyrosinase (24) and gp100 (25). The elution of peptides from specific MHC class II molecules can be cumbersome and requires selection of specific MHC restriction elements for study. Other groups have identified Th epitopes by screening panels of overlapping peptides derived from the antigen being studied using human lymphocytes (*see* Table 3). Class II binding epitopes from tetanus toxoid (26), diphtheria toxin (27), as well as the MAGE-3 melanoma tumor antigen (28) and Kallikrein 4, a prostate cancer antigen (29), have been identified by this method. In the case of tetanus and diphtheria toxins, panels of peptides of varying lengths were used, spanning the entire length of the proteins, as stimulator antigens in T-cell proliferation assays to determine which peptides stimulated proliferation using lymphocytes derived from antigen-immune individuals. The advantage to this type of approach is that the results are exhaustive, spanning the entire length of the immunogen. A significant disadvantage is the cost of constructing peptides overlapping an entire antigen.

Similar to the identification of class I binding motifs, computer algorithms have played a major role in the identification of class II binding motifs. Class II antigenic epitopes tend to share a common secondary structure, namely amphipathic α helices (30), suggesting antigenic epitopes recognized by T cells can be predicted from the primary structure of a protein based on predicted secondary structure (31,32). There are now several amphi-

pathicity and hydrophobicity algorithms that have been demonstrated to be useful in predicting T-cell presented sequences within potentially antigenic proteins (30,31,33). These algorithms have been used to identify potential MHC class II-binding epitopes from tumor-associated antigens. In vitro generation of peptide specific T cells from patients with cancer, similar to those used with class I peptides, is the end point of the identification of epitopes appropriate for use in immunization. This strategy was used to define putative class II HER-2/neu-binding epitopes for use in a cancer vaccine.

HER-2/neu peptides, with the potential for eliciting an immune response, were initially selected using a computer protein sequence analysis package using searching algorithms for identifying motifs according to charge and polarity patterns and tertiary structure, particularly related to amphipathic alpha helices (31). Each of the searching algorithms had empirically been successful in identifying a substantial proportion (50–70%) of Th cell epitopes in foreign proteins (34,35). Analysis resulted in the identification of more than 40 HER-2/neu peptides with a high potential to interact with human class II (1). Based on predicted interactions, 26 peptides, 15–18 amino acids in length, were constructed. Seven of 26 peptides tested demonstrated the ability to elicit T-cell responses, in vitro, in at least some of breast cancer patients evaluated (1). When used in active immunization, four of the seven peptides generated detectable immunity in greater than two-thirds of patients immunized. Subsequently, when these peptides were tested for binding to HLA-DR molecules in vitro, the peptides that were the most immunogenic in vivo were also those peptides that had the highest binding affinity to HLA-DR (36).

Identification of class II epitopes may be further facilitated by screening identified peptides using lymphocytes derived from cancer patients who have a preexistent immune response to the candidate tumor antigen. The epitopes of prostatic acid phosphatase (PAP), a prostate cancer antigen, were defined by evaluating T-cell responses in patients with or without a preexistent T-cell response to PAP (37). Four PAP-specific Th peptides were identified for which peptide-specific T-cell proliferation occurred in the majority of patient samples that also exhibited PAP-specific T-cell proliferation. Thus, over the last several years the identification of class II binding epitopes to common tumor antigens has been facilitated by improvements in the ability to predict class II binding as well as the development of techniques for rapid in vitro screening of immune responses.

3.3. Patients With Cancer Can Be Immunized With Class II Peptide-Based Vaccines

A potential pitfall of the use of single class I binding peptides is illustrated in two similar studies vaccinating patients with an HLA-A2-binding peptide, p369–377, derived from the protein sequence of HER-2/neu, a well-defined tumor antigen. In an initial clinical study, HLA-A2-positive patients with metastatic HER-2/neu-overexpressing breast, ovarian, or colorectal carcinomas were immunized with 1 mg of p369–377 admixed in incomplete Freund's adjuvant (IFA) every 3 wk (38). Peptide-specific CTLs were isolated and expanded from the peripheral blood of patients after two or four immunizations. The CTLs could lyse HLA-matched peptide-pulsed target cells but could not lyse HLA-matched tumors expressing the HER-2/neu protein. Even when tumors were treated with IFN- γ to upregulate class I, the CTL lines generated from the patients would not respond to the peptide presented endogenously on tumor cells. More recently a similar study was performed, immunizing patients with p369–377 using GM-CSF as

an adjuvant (39). GM-CSF is a recruitment and maturation factor for skin dendritic cells (DCs), Langerhans cells (LCs), and theoretically may allow more efficient presentation of peptide epitopes than standard adjuvants such as IFA. Six HLA-A2 patients with HER-2/neu-overexpressing cancers received six monthly vaccinations with 500 µg of HER-2/neu peptide, p369–377, and mixed with 100 µg of GM-CSF. The patients had either stage III or IV breast or ovarian cancer. Immune responses to the p369–377 were examined using an IFN- γ ELIspot assay. Prior to vaccination, the median precursor frequency, defined as precursors/10⁶ PBMC, to p369–377 was not detectable. Following vaccination, HER-2/neu peptide-specific precursors developed to p369–377 in just two of four evaluable subjects. The responses were short-lived and not detectable at 5 mo after the final vaccination. Immunocompetence was evident as patients had detectable T-cell responses to tetanus toxoid and influenza. These results demonstrate that HER-2/neu MHC class I epitopes can induce HER-2/neu peptide-specific IFN- γ -producing CD8⁺ T cells. However, the magnitude of the responses were low, as well as short-lived. Theoretically, the addition of CD4⁺ T-cell help would allow the generation of lasting immunity.

A successful vaccine strategy for generating peptide-specific CTLs capable of lysing tumor expressing HER-2/neu and resulting in durable immunity involved immunizing patients with putative Th epitopes of HER-2/neu that had, embedded in the natural sequence, HLA-A2-binding motifs of HER-2/neu. Thus, both CD4⁺ T-cell help and CD8⁺-specific epitopes were encompassed in the same vaccine. In this trial, 19 HLA-A2 patients with HER-2/neu-overexpressing cancers received a vaccine preparation consisting of putative HER-2/neu helper peptides (40). Contained within these sequences were the HLA-A2-binding motifs. Patients developed both HER-2/neu-specific CD4⁺ and CD8⁺ T-cell responses. The level of HER-2/neu immunity was similar to viral and tetanus immunity. In addition, the peptide-specific T cells were able to lyse tumor. The responses were long-lived and detectable for greater than 1 yr after the final vaccination in selected patients. These results demonstrate that HER-2/neu MHC class II epitopes containing encompassed MHC class I epitopes are able to induce long-lasting HER-2/neu-specific IFN- γ -producing CD8 T cells. Currently, several groups are focusing on the identification of peptides that are suited for binding to class II and contain within their natural sequence class I epitopes for the same antigen. A peptide with dual class I and II specificities has recently been identified for NY-ESO (41) as well as the melanoma antigen, gp100 (42).

Stimulating an effective Th response, even without concomitant CD8⁺ peptide vaccination, is a way to boost antigen-specific immunity as CD4⁺ T cells generate the cytokine environment required to support an evolving immune response. Vaccinating patients with HER-2/neu Th peptides, patients with advanced-stage HER-2/neu-overexpressing breast, ovarian, and non-small cell lung cancer were enrolled. Thirty-eight patients finished the planned course of six immunizations (2). Patients received 500 µg of each peptide admixed in GM-CSF (43). Over 90% of patients developed T-cell immunity to HER-2/neu peptides and over 60% to a HER-2/neu protein domain. Thus, immunization with peptides resulted in the generation of T cells that could respond to protein processed by APCs. Furthermore, at 1-yr follow-up, immunity to the HER-2/neu protein persisted in over a third of patients. Immunity elicited by active immunization with CD4⁺ T-helper epitopes was durable. An additional finding of this study was that epitope spreading was observed in the majority of patients and significantly correlated

with the generation of HER-2/neu protein-specific T-cell immunity. Epitope, or determinant spreading, is a phenomenon first described in autoimmune disease (44) and represents the generation of an immune response to a particular portion of an immunogenic protein and then the natural spread of that immunity to other areas of the protein or even to other antigens present in the environment. The same phenomenon, epitope spreading, was reported recently in a vaccine trial immunizing breast and ovarian cancer patients with autologous DCs pulsed with MUC-1 or HER-2/neu peptides (45). Epitope spreading may represent the ability of the initial immune response to create a microenvironment at the site of the tumor that enhances endogenous local immunity (46). Despite the generation of detectable immunity against the self-protein HER-2/neu and the development of epitope spreading, none of the patients in the studies described above developed any evidence of autoimmunity directed against tissues expressing basal levels of HER-2/neu such as skin, liver, and digestive tract epithelium.

4. MODIFICATIONS OF PEPTIDES TO IMPROVE IMMUNOGENICITY

Initial clinical trials of peptide-based vaccines indicated that patients can be immunized, but immune responses were generally not to the levels of a vaccinated antigen such as tetanus toxoid. Therefore, more recent investigations are focused on improving the immunogenicity of peptide immunization either by modifying the route, adjuvant, or dose of the vaccine or via the modification of the peptide itself.

4.1. Practical Issues of Peptide Immunization Assessed in Early Human Clinical Trials

One of the first considerations addressed in initial clinical trials of peptide-based vaccines was the route of administration. The primary routes of administration that have been used are the intramuscular, subcutaneous, and intradermal routes. The dose and volume of the vaccine itself, the choice of adjuvant, and the desired immune response have largely determined immunization route. There have been few human clinical peptide vaccine studies comparing the routes of administration. For example, one reported study used both an intramuscular and subcutaneous route for vaccination but did not have the power to detect a statistically significant difference in immune response (47). Early vaccine studies with peptide and protein subunit vaccines primarily used an intramuscular approach, based on the model of vaccination for infectious diseases (48–50). Most studies demonstrated evidence of antigen-specific antibody production, and one study showed evidence of a concomitant Th response (50). Clinical investigations using infectious-disease vaccines have given an indication of the most effective routes of immunization by comparing the intramuscular and intradermal routes of immunization for the recombinant hepatitis B surface antigen whole-protein vaccine. Investigators found the intradermal route superior with efficient production of antigen-specific Th cells, antibodies, and CTLs. In addition, patients who were not immunized by the intramuscular route of injection could subsequently be effectively immunized by vaccinating via the intradermal route (51). Furthermore, investigations in a rodent model have demonstrated that the intradermal route was preferable to the subcutaneous route in initiating a T-cell response, presumably due to the presence of professional antigen-presenting LCs in the

skin (43). Thus, a majority of peptide vaccine trials have predominantly used subcutaneous and intradermal routes of immunization (52–56), the subcutaneous route having been chosen by some based on prior animal studies and choice of adjuvant (52,57) and by others to accommodate larger volumes of administered vaccine (56).

The choice of adjuvant is another consideration studied in peptide vaccine trials, particularly as peptides themselves are typically only weakly immunogenic. Due to recent renewed enthusiasm for peptide-based tumor vaccines coupled with the search for adjuvants that promote cellular as well as antibody responses, the list of adjuvants being studied continues to grow. The choice of adjuvant has largely been determined by pre-clinical models. Similar to alum, oil-based incomplete Freund's-type adjuvants, such as Montanide ISA-51 (Seppic, Paris, France) and TiterMax (CytRx Corp., Norcross, GA), have been used in human peptide vaccine studies (56,58), with their adjuvant effect likely mediated by a depot effect, increasing the half-life of the peptide antigen at the site of immunization. Other pro-inflammatory biologic adjuvants such as BCG, Detox (mycobacterial cell-wall skeleton plus a lipid moiety) (Corixa Corp., Seattle, WA), and influenza virosomes have been used as adjuvants in peptide- and protein-based human vaccine studies (59–61). One study compared a variety of adjuvants in a rodent model targeting human MUC1 peptide antigens conjugated to keyhole limpet hemocyanin (KLH), and found the saponin adjuvant QS-21 (Aquila Biopharmaceuticals, Worcester, MA) to be the most effective in promoting an antigen-specific antibody responses (62). Subsequently, QS-21 has been used as an adjuvant in human peptide cancer vaccine trials (57,63), with the hope that the use of a saponin may allow entry of peptides directly into MHC complexes to stimulate a T-cell response (64). Another type of adjuvant that has been studied in animal models is polymer microspheres that encapsulate the peptide antigen, permitting slow release over variable lengths of time (65). Potential advantages to this type of adjuvant include the ability to use an oral route of delivery (66), continuous release of antigen obviating the need for booster immunizations, and the ability to incorporate other biologic adjuvants or cytokines within the polymer (67). Human trials using microsphere adjuvants in the context of peptide-based cancer vaccines are under way. Finally, the discovery of various cytokines participating in the initiation of immune responses has suggested that cytokines themselves may be useful immunologic adjuvants. As an example, soluble GM-CSF is a vaccine adjuvant, and has the ability to induce the differentiation of DCs and act as a chemoattractant for various immune cell effectors (68,69).

The dose of peptide administered is another factor that has been extensively studied in clinical peptide vaccine trials. Over the last several years that peptide vaccines have been in clinical use, there have been multiple-dose escalation studies designed to evaluate the immunogenicity of progressively higher doses of peptide (50,52,56–58,70). It is difficult to draw overall conclusions from these studies, however, because length and/or stability of peptide, routes of administration, choice of adjuvant, dose, and analysis of immunologic response vary greatly from study to study. In addition, patient cohorts were generally too small to draw definitive conclusions regarding dose efficacy. No peptide dose-related toxicities have been observed, even up to doses of 2000 µg of a 9-mer peptide (56). Toxicities that have been observed were not related to dose of peptide, but rather were a consequence of the adjuvant used (58,70). Regarding immune responses, two studies suggest that doses of <100 µg administered subcutaneously were less effec-

tive than higher doses (52,57). Two studies with peptide administered intramuscularly, however, found transient immune responses at even the lowest doses of 10 µg used (50,70). Several studies showed little difference in immune responses to peptide doses >100 µg (50, 56,57). Clinical vaccine trials in multiple sclerosis have shown that immunization with T-cell receptor (TCR) peptides in doses >1000 µg could produce a tolerizing Th2 peptide-specific response with clinical response (71,72). These results suggest that peptide doses of 100–1000 µg may be appropriate for human peptide vaccines targeting cancer-associated antigens. In addition, because peptides themselves have not to date demonstrated considerable toxicity and are unlikely to exert a biologic effect in the same way as pharmacological agents, the paradigm of dose-escalation studies in phase I trials should perhaps be different. Rather than determining the maximal tolerated dose, a better goal may be to establish the minimal dose required to generate an effective immune response.

4.2. Modification of Binding Sites

The relationship between class I affinity and tumor antigen epitope immunogenicity is of importance because tissue-specific and developmental tumor antigens, such as those that are tumor antigens, are expressed on normal tissues at some point in time at some location within the body. T cells specific for these self-antigens could be functionally inactivated by T-cell tolerance. However, CTL respond to tumor epitopes in both volunteer donors and cancer patients, indicating that tolerance to these tumor antigens, if it exists at all, is incomplete (5,73). Whether or not T cells recognizing high-affinity epitopes, have been selectively eliminated, leaving a repertoire capable of recognizing only low-affinity epitopes, is not known. One investigation evaluated several peptides derived from four different tumor antigens, p53, HER-2/neu, CEA, and MAGE proteins, for their capacity to induce CTL *in vitro* capable of recognizing tumor target lines (5). In order to increase the likelihood of overcoming tolerance, fixed-anchor analogs that demonstrate improved HLA-A*0201 affinity and binding were used. Forty-two wild-type and analog peptides were screened. All the peptides bound HLA-A*0201 and two or more additional A2 supertypes alleles with an IC₅₀ of 500 nM or less. A total of 20 of 22 wild-type and 9 of 12 single amino acid substitution analogs were found to be immunogenic in primary *in vitro* CTL induction assays, using normal peripheral blood mononuclear cells (PBMCs) and monocyte-derived DCs as APCs. CTLs generated by 13 of 20 of the wild-type epitopes and 6 of 9 of single-substitution analogs tested recognized HLA-matched antigen-bearing cancer cell lines. Further analysis revealed that recognition of naturally processed antigen was correlated with high HLA-A2.1-binding affinity (IC₅₀ = 200 nM or less; $p=0.008$), suggesting that high-binding-affinity epitopes are frequently generated and can be recognized as a result of natural antigen processing. Indeed, the immunogenicity of CTL epitopes derived from human tumor antigens such as CEA can be improved by altering the peptide motif to improve binding to MHC (74). Studies such as these demonstrate that recognition of self-tumor antigens is within the realm of the T-cell repertoire and that binding affinity may be an important criterion for epitope selection.

5. CONCLUSION

The definition of multiple tumor antigen-specific peptides, both class I- and class II-derived epitopes, are rapidly being translated into human clinical studies. Investigations over the last decade have allowed the development of technology such as computer algorithms and transgenic mice to rapidly screen vaccine candidates. Clinical trials of single peptides have demonstrated that cancer patients can be immunized against self-tumor antigens and some studies have shown early positive clinical results. Current effort in the field is focused on improving the immunogenicity of individual MHC-binding peptides as well as developing multiple peptide vaccines for the prevention and treatment of human malignancy.

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11

Antibody-Inducing Cancer Vaccines Against Cell-Surface Carbohydrate Antigens

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1. THE RATIONALE FOR POLYVALENT ANTIBODY-INDUCING CANCER VACCINES AGAINST CARBOHYDRATE ANTIGENS

1.1. Introduction

Carbohydrate cell-surface antigens have proved to be unexpectedly potent targets for immune recognition and attack against cancers (reviewed in 1). Of the many tumor-restricted monoclonal antibodies derived by immunization of mice with human tumor cells, most have been directed against carbohydrate antigens expressed at the cell surface as glycolipids or mucins (2–4). Antibodies against cell-surface antigens such as these are ideally suited for eradication of free tumor cells and micrometastases. This is the role of antibodies against most infectious diseases and it has been accomplished against cancer cells as described below in a variety of preclinical models. In adjuvant immunization trials, the primary targets are individual tumor cells or early micrometastases, which may persist for long periods after apparent resection of all residual tumor (5–7). After surgery

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and completion of chemotherapy is the ideal time for immune intervention, and in particular for administration of cancer vaccines aimed at instructing the immune system to identify and kill these few remaining cancer cells. If antibodies of sufficient titer can be induced against tumor antigens to eliminate tumor cells from the blood and lymphatic systems, and to eradicate micrometastases (making establishment of new metastases no longer possible) this would dramatically change our approach to treating the cancer patient. Aggressive local therapies, including surgery, radiation therapy, and intralesional treatments, might result in long-term control of even metastatic cancers.

In fact, antibodies have demonstrated antitumor efficacy *in vivo*:

1. There are many preclinical models demonstrating that passively administered or actively induced antibodies (generally against carbohydrate antigens) can prevent tumor recurrence (reviewed in refs 8 and 9).
2. There are an increasing number of clinical trials where passively administered monoclonal antibodies (MAbs) have demonstrated clinical efficacy.
3. Naturally acquired or vaccine induced antibodies against cancer cell–surface antigens, especially carbohydrate antigens, have correlated, with improved prognosis in several different clinical settings (reviewed in refs. 10–13).

1.2. Polyvalent Vaccines

The basis for emphasis on polyvalent vaccines is tumor cell heterogeneity, heterogeneity of the human immune response, and the correlation between overall antibody titer against tumor cells and effector mechanisms such as complement-dependent cytotoxicity (CDC) or antibody-dependent cell-mediated cytotoxicity (ADCC). For example, using a series of 14 tumor cell lines and MAbs against three gangliosides, we have shown that significant cell-surface reactivity analyzed by flow cytometry and CDC increased from two to eight of the cell lines using one of three MAbs to all 14 of the cell lines when the three MAbs were pooled. The median CDC increased fourfold with the pool of MAbs compared to the best single MAb (14).

1.3. Treatment in the Adjuvant Setting

The basis for emphasis on vaccination in the adjuvant setting is best demonstrated in preclinical models. The syngeneic murine tumor models involving EL4 lymphoma are particularly informative in terms of trial design (9). EL4 lymphoma naturally expresses GD2 ganglioside, which is recognized by MAb 3F8. Vaccines containing GD2 covalently conjugated to keyhole limpet hemocyanine (KLH) and mixed with immunological adjuvant QS21 are optimal for vaccination against GD2. Relatively higher levels of MAb administered 2 or 4 d after intravenous tumor challenge or moderate titers induced by vaccination that were present by day 4 after tumor challenge were able to eradicate disease in most mice. If MAb administration was deferred until day 7 or 10 after iv challenge, little or no benefit could be demonstrated. If the number of cells in the EL4 challenge was decreased, giving a longer window of opportunity, the vaccinations could be initiated after tumor challenge and good protection seen (9). These results are consistent with the need to initiate immunization with vaccines inducing antibodies in the adjuvant setting, when the targets are circulating tumor cells and micrometastases.

Comparable benefit is also seen when a subcutaneous foot-pad tumor challenge model, which more closely mirrors the clinical setting, is used (*see* Fig. 1). Vaccination or MAb

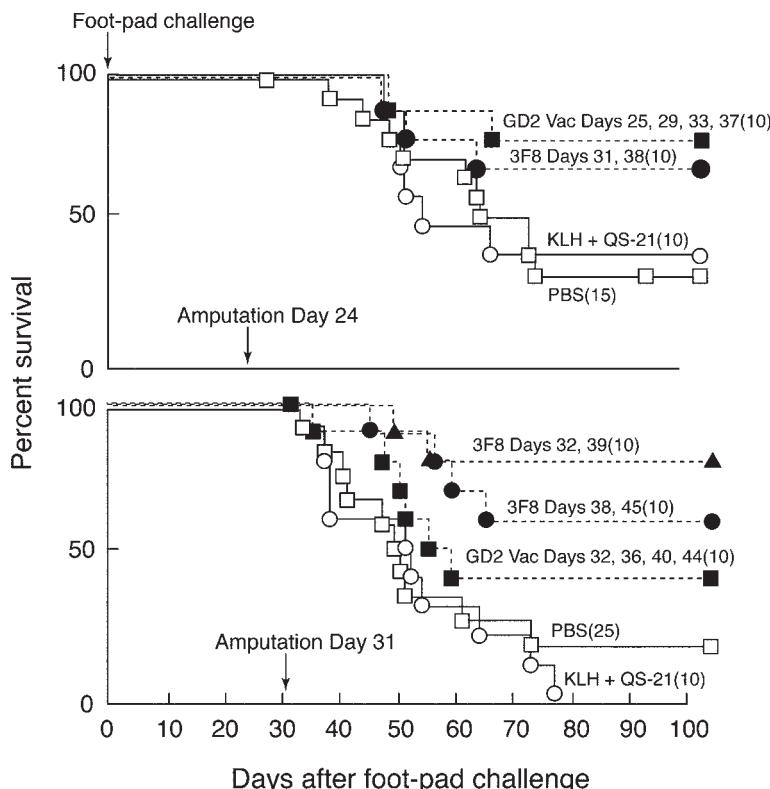


Fig. 1. Survival of groups of 10 mice challenged in the foot-pad with 10^5 EL4 lymphoma cells on day 0. The foot was amputated on day 24 or 31, and mice were treated after amputation with GD2-KLH plus QS-21 vaccine, 3F8 MAb, or various negative control treatments.

administration after amputation of the foot-pad primary tumor results in cure of most mice. There are comparable syngeneic models demonstrating the antitumor efficacy of MAbs or vaccines against other glycolipids (GD3, GM3), mucin antigens (Tn, TF, and MUC1) and a protein antigen (gp75) (reviewed in 1 and 2). These trials all share one thing in common: Benefit is seen primarily in minimal disease settings, comparable to the adjuvant setting in the clinic.

1.4. Advantages of Vaccines That Induce Antibodies Over Vaccines Designed to Augment T-Lymphocyte Immunity

The concept of a vaccine that consistently augments T-lymphocyte immunity against cancer cells is exciting and offers enormous potential for clinical benefit. However, demonstration that this has been achieved has proven to be more difficult than initially appreciated, for a variety of reasons:

1. Ideally, autologous cancer cells are required for testing and these are rarely available as cell lines or in frozen samples in sufficient quantities for a thorough analysis of the immune response and its specificity.
2. In vitro sensitization has in the past generally been required for demonstration of T-cell responses against tumor antigens and this adds significant risk of artifactual results and complicates the quantification of immune responses.

3. Augmentation of T-cell responses by vaccination in humans is more difficult to induce than augmentation of B-cell responses and has yet to be clearly achieved and confirmed in a majority of vaccinated patients against any tumor antigen.
4. Vaccine design depends on the immune response desired. There are hundreds of available approaches or combinations of approaches to inducing T-cell immunity. These include immunization with peptides or proteins with various adjuvants, dendritic cells pulsed with or transduced to express particular antigens, viruses or bacteria transduced to express antigens, and DNA or RNA vaccines. In each case these vaccines could include approaches to augmenting cytokine or second-signal induction. The range of options for augmenting T-cell immunity against cancer is daunting. Unlike the picture with vaccines designed to induce an antibody response where there is one best approach (conjugate vaccines as described below), it remains unclear which is the optimal approach for induction of T-cell immunity.
5. It is unclear whether augmentation of cytotoxic T lymphocytes (CTLs) or helper T cells is the desired goal for vaccines inducing T-cell immunity against cancer.
6. It is not clear which antigens should be selected as targets for T-cell attack against cancer, as no T-cell immune responses have been correlated with a more favorable prognosis as is true for antibody responses against glycolipids (GM2) and mucins (sTn) (10–12,51).
7. Tumor cells can and frequently do fail to express relevant antigens in the context of major histocompatibility complex (MHC) as a consequence of MHC loss or problems in antigen processing (proteasomes, TAP), or they may suppress the T-cell response or become resistant to it (by production of IL-10, TGF- β , VEGF, Fas-ligand, HLA-G, or Bcl-2) (reviewed in refs. 52 and 53).

Given these uncertainties, selection of a single vaccine approach for inducing optimal T-cell immunity, unlike the situation with antibody-inducing vaccines, is difficult now and will remain so for some years to come. Consequently, we have focused on antibody-inducing polyvalent vaccines targeting primarily the carbohydrate antigens listed in Table 1 plus a few glycoprotein antigens such as MUC1 and KSA (also termed EpCam) (54–58) in epithelial cancers, PSMA in prostate cancers, and CA125 (now termed MUC16) in ovarian cancers (59).

2. SELECTION OF CELL-SURFACE CARBOHYDRATE ANTIGENS AS TARGETS FOR IMMUNE ATTACK AGAINST CANCER

2.1. *Carbohydrate Cell-Surface Cancer Antigens: The MSKCC Experience*

We have screened a variety of malignancies and normal tissues with a series of 40 monoclonal antibodies against 25 antigens that were potential target antigens for immunotherapy (18–21). Results for the 12 defined antigens expressed strongly in 50% or more of biopsy specimens of breast, ovary, prostate cancer, melanoma, sarcoma, and small-cell lung cancer (SCLC) are shown as examples in Table 2. The 13 excluded antigens (including CEA [carcinoembryonic antigen] and HER2/neu) were expressed in 0–2 of the 5–10 specimens.

With the exception of MUC1 and KSA, all of the widely expressed antigens on these cancers were carbohydrates. Our results are consistent with those from other centers with one exception: We did not find increased levels of GD2 or GD3 in SCLC. There is a striking similarity in expression of these 12 antigens among tumors of similar embryologic background (i.e., epithelial vs neuroectodermal). Epithelial cancers (breast, ovary,

Table 1
Carbohydrate Cancer Cell-Surface Targets for Vaccine Construction

Tumor	Antigens ^a
Melanoma	GM2, GD2, GD3
Neuroblastoma	GM2, GD2, GD3, polysialic acid
Sarcoma	GM2, GD2, GD3
Small-cell lung cancer	GM2, fucosyl GM1, polysialic acid, globo H, sialyl Le ^y
Breast	GM2, globo H, Le ^y , TF, Tn, sTn
Prostate	GM2, Tn, sTn, TF, Le ^y
Ovary	GM2, globo H, sTn, TF, Le ^y

^aAntigens present on at least 50% of cancer cells in at least 50% of biopsy specimens.

Table 2
Proportion of Tumor Specimens With 50% or More of Cells Positive by Immunohistology

Antigen	sTn	Tn	TF	Globo H	Le	GM2	GD2	GD3	FUC GM1	Polysialic acid
	MAb	CC49	IE3	49H.8	MBr1	BR96	696	3F8	R24	F12
Tumor										
Breast	5/10 ^a	5/10	6/10	4/5	7/10	5/5	0/5	0/5	0/5	0/6
Ovary	4/5	1/5	5/5	3/5	5/5	5/5	0/5	0/5	0/5	0/5
Prostate	6/11	10/11	10/11	2/11	4/11	11/11	0/5	0/5	0/5	0/5
Melanoma	0/5	0/5	0/5	0/10	0/5	10/10	6/10	8/10	0/10	0/6
Sarcoma	0/5	0/5	0/5	0/5	0/5	8/9	5/9	4/9	0/0	1/5
Small-cell lung cancer	0/5	0/5	0/5	4/6	2/5	6/6	0/6	0/6	4/6	6/6

^aNumber of tumor specimens positive/number of tumor specimens tested.

prostate colon, etc.) but not cancers of neuroectodermal origin (melanomas, sarcomas, neuroblastomas) expressed MUC1, Tn, sTn, TF, globo H, and Lewis^y (Le^y), whereas only the neuroectodermal cancers expressed GD2 and GD3. SCLC shared some characteristics of each and in addition expressed fucosyl GM1 and long chains of poly- α 2,8-sialic acid, which were not expressed in tissues of either background.

2.2. Gangliosides GM2, GD2, GD3, and Fucosyl GM1

Gangliosides are sialic acid-containing glycolipids that are expressed at the cell surface with their lipid (ceramide) moiety incorporated into the cell-surface lipid bilayer. Most gangliosides considered as potential targets for cancer therapy are expressed primarily in tissues and tumors of neuroectodermal origin. This is true for the melanoma, sarcoma, neuroblastoma, and SCLC antigens GM2, GD2, and GD3, and the SCLC antigen fucosyl GM1. The structures of these antigens are shown in Fig. 2. Surprisingly, however,

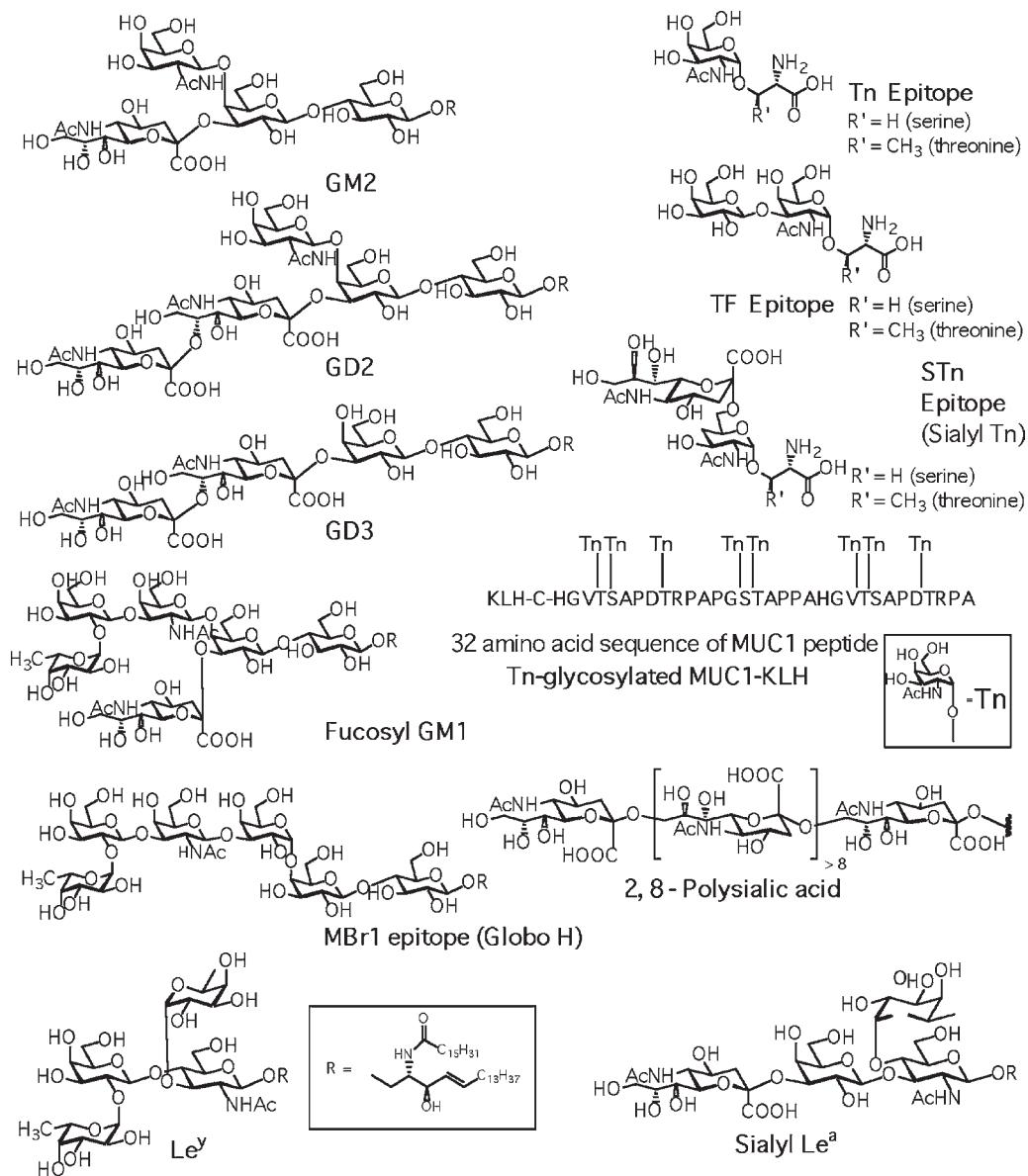


Fig. 2. Carbohydrate antigens expressed at the cell surface of human cancers that are targets for antibody-inducing cancer vaccines.

GM2 has also recently been identified in a number of epithelial cancers (22,23) and at the luminal surfaces of a variety of normal epithelial tissues.

2.3. Neutral Glycolipids Lewis^y and Globo H

Le^y and Globo H antigens are found at the cell surface of epithelial cancers primarily expressed as glycolipids attached to the lipid bilayer by hydrophobic forces through the ceramide, but they are also *O*-linked via -OH groups of serine or threonine to mucins and

N-linked via the NH₂ group of asparagine in other proteins (21–24). Whether expressed as glycolipids or glycoproteins, the immune response against these antigens is predominantly against the carbohydrate moiety. The expression of Le^y, Le^a, and Globo H on various types of cancer cells has been well documented (24–27). They are expressed in lesser amounts on a variety of normal tissues, again at the lumen border of ducts and in secretions as described for TF and sTn (2,4,19). Monoclonal antibodies against each have shown good localization to human cancers *in vivo* (28,29). The structures of these antigens in their glycolipid form are shown in Fig. 2.

2.4. *TF, Tn, and sTn Antigens*

Mucins are major cell-surface antigens in a variety of epithelial cancers. They are primarily large extracellular molecules made up of multiple copies of serine- and threonine-rich tandem repeats (30–33). Though mucins (including carbohydrate and peptide epitopes) are also expressed on some normal tissues, they have proved to be excellent targets for anticancer attack for two reasons: First, expression on normal tissues is largely restricted to the ductal border of secretory cells (31–33), a site largely inaccessible to the immune system. Cancer cells, on the other hand, have no patent ducts and so accumulate mucins over the entire cell surface. Second, peptide backbones of cancer mucins are not fully glycosylated and glycosylation that does occur is not complete. Glycosylation of cancer mucins with mono- or disaccharides such as Tn, sTn, or TF *O*-linked to serines or threonines is especially common. Thomsen-Friedenreich antigen (TF; Galβ1-3GalNAcα-O-serine/threonine), Tn (GalNAcα1-O-serine/threonine), and sialyl Tn (sTn; NANAα2-GalNAcα1-O-serine/threonine) are monosaccharide or disaccharide antigens expressed *O*-linked to mucins in a variety of epithelial cancers (34,35). Expression of these mono- and disaccharides correlates with a more aggressive phenotype and a more ominous prognosis (36,37). TF, Tn, and sTn are expressed in 50–80% of various epithelial cancers (38–40). STn trimer (cluster) is the epitope recognized by monoclonal antibody B72.3, and TF and sTn are, or are closely associated with, the clustered epitope recognized by monoclonal antibody CC49 (41). Clinical trials of radiolabeled CC49 administered intraperitoneal (ip) in patients with breast cancer (42) and ovarian cancer (43) at this center and elsewhere have shown excellent targeting. TF has also been used successfully as a target for cancer imaging (44). TF, Tn, and sTn are expressed to a lesser extent on a variety of normal tissues, where they are expressed predominantly as occasional monomers at luminal surfaces (19,45). Immunohistology performed with MAbs identifying these trimers react strongly with a variety of epithelial cancers but only minimally with normal tissues, suggesting that focusing on the trimers of Tn, sTn, and TF further increases the tumor specificity of the immune response. Immunization with TF and Tn has been shown to protect mice from subsequent challenge with syngeneic cancer cell lines expressing these antigens (46,47). Hence both active and passive immunotherapy trials have identified TF, Tn, and sTn antigens as uniquely effective targets for cancer targeting and immunotherapy.

2.5. *Polysialic Acid*

The neural cell adhesion molecule (N-CAM) is expressed on the cell surface of embryonic tissues, neuroendocrine cells, and a variety of neuroendocrine tumors including SCLC, neuroblastomas, and carcinoids (48,49). N-CAM undergoes a series of post-translational modifications, with the acquisition of \approx 2,8-linked sialic acid residues as

long polysialic acid chains (20–100 residues). Several monoclonal antibodies, including MAb 735 and NP-4, recognize these long polysialic acid chains (50) and have allowed characterization of this antigen in both normal and malignant tissue. Zhang et al. has demonstrated that of six SCLC tumor specimens, all were reactive by immunohistochemistry using MAb 735, and five of the six tested SCLC tumor specimen were positive using MAb NP-4 (18). This confirms previous results of Komminoth (49), and suggests that polysialic acid may serve as a useful target for immune attack against SCLC. Polysialic acid is also expressed in the gray matter of the brain, bronchial epithelia and pneumocytes, epithelia of the colon, stomach, and pancreas, and capillary endothelial cells and ganglion neurons in the colon. The reactivity of these antibodies in epithelia is restricted to the luminal surfaces of glandular tissues, where access to the immune system is restricted. Two to 5% of normal donors have high levels of antibody against polysialic acid as a consequence of exposure to bacteria such as *Neisseria meningitidis* group B (MenB) and *Escherichia coli* K1 that also express polysialic acid. This has not been associated with any signs of autoimmunity. Consequently, vaccines against polysialic acid are being tested to combat these diseases; however, polysialic acid has proven to be poorly immunogenic.

With few exceptions (MUC1, CEA, and KSA on a variety of epithelial cancers; CA125 on ovarian cancers and PSMA on prostate cancers), other antigens are not as abundantly expressed, nor are they expressed with the same high frequency on cancers from different patients as are the carbohydrate antigens described above. In addition, antigens such as the cancer-testis antigens and p53 are not cell-surface antigens, which may restrict the relevant immune response to a T-cell response. This enormously complicates the analysis of immunogenicity in vaccine trials for the reasons described above. Consequently, we have focused on antibody-inducing polyvalent vaccines targeting primarily the carbohydrate antigens listed in Table 1 plus a few glycoprotein antigens such as MUC1 and KSA (also termed EpCam) (54–58) in epithelial cancers, prostate-specific membrane antigen (PSMA) in prostate cancers, and CA125 (now termed MUC16) in ovarian cancers (59).

3. IMMUNOGENICITY OF THESE CELL-SURFACE CARBOHYDRATES IN CANCER PATIENTS

3.1. Selection of KLH Conjugate Plus QS-21 Vaccines

We have explored a variety of approaches for increasing the antibody response against carbohydrate and peptide cancer antigens, including the use of different immunological adjuvants (12,60–65), chemical modification of gangliosides to make them more immunogenic (66–69), and conjugation to various immunogenic carrier proteins (60,70). The conclusion from these studies is that the use of a carrier protein plus an immunological adjuvant is the optimal approach. The optimal immunological adjuvant in each case was one or more purified saponin fractions (QS-21 or GPI-0100) obtained from the bark of *Quillaja saponaria* (65,71). The optimal carrier protein was in each case KLH. This approach (covalent attachment of the carbohydrate to KLH and administration mixed with QS-21 or GPI-0100) has proved optimal for antibody induction in mice and cancer patients for each of the antigens in Table 1, except for sLe^a, which has not been tested yet. The role of carrier protein in these conjugate vaccines is to induce potent T-lymphocyte help against the carrier (KLH), which also provides help for the antibody response against any covalently attached molecules such as these tumor antigens.

Two additional variables have proved critical for increasing antibody titers, the method of conjugation and the epitope ratio of antigen molecules per KLH molecule. The optimal conjugation approached has varied with the antigen. Gangliosides are best conjugated using ozone cleavage of the ceramide double bond and introducing an aldehyde group followed by coupling to aminolysyl groups of KLH by reductive amination. This approach was not as effective for conjugation of Tn, sTn, and TF clusters or Globo H to KLH where an M2C2H linker arm has proved most efficient (77) or for MUC1 or MUC2 where an MBS linker group was optimal (60). We have demonstrated that covalent conjugation of antigen (ganglioside GD3) to KLH is required; simply mixing the two is of little benefit (70). Based on our experience with GM2 and GD3 conjugate vaccines, it is our impression that within the restrictions imposed by current conjugation methods, higher epitope ratios result in higher immunogenicity. Consequently considerable effort must be devoted to optimizing this ratio with each vaccine.

We have also performed a series of phase I dosing trials to determine the impact of dose of conjugate on antibody response in vaccinated patients, and a series of experiments to determine the impact of treatments designed to decrease suppressor-cell reactivity in mice. The lowest dose of antigen in the KLH conjugates resulting in optimal antibody titers for each antigen is listed in Table 3. The lowest optimal doses range from 1 µg for TF to 10 µg for the glycolipids. Decreasing suppressor-cell activity with low-dose cyclophosphamide or anti-CTLA4 MAb had no impact on antibody titers.

3.2. *Ganglioside Vaccines*

We have been refining our ability to induce antibodies against GM2 in melanoma patients for 15 yr, since it was first demonstrated that patients immunized with irradiated melanoma cells occasionally produced antibodies against GM2, and that vaccines containing purified GM2 could be more immunogenic than vaccines containing tumor cells expressing GM2 (72). Initially GM2 adherent to bacille Calmette-Guérin (BCG) was selected as optimal, inducing immunoglobulin M (IgM) antibody responses in 85% of patients. Antibody responses are defined here as an ELISA titer of 1/40 or greater (or at least eightfold above baseline) confirmed by reactivity against cancer cells by immune thin-layer chromatography or flow cytometry. Though these antibodies and monoclonal antibodies against GM2 were only able to kill 25% of melanoma cell lines by CDC, patients with natural or vaccine-induced antibodies had significantly longer disease-free and overall survival (11). This was the basis for a randomized trial comparing immunization with BCG to immunization with GM2/BCG in 122 patients with AJCC stage 3 melanoma (12). Though the difference was not statistically significant, the GM2/BCG-treated patients had a 12% improvement in survival and 15% improvement in disease-free survival compared to the BCG patients after a minimum follow-up of 70 mo. The IgM antibodies had a median titer of 1/160 and were short lived (8–12 wk). Immunoglobulin G (IgG) antibody induction was rare. We explored a variety of approaches to further improve this antibody response (70). The use of GM2 conjugated to KLH and mixed with immunological adjuvant QS-21 was consistently optimal, inducing higher titer IgM antibodies (median titer 1/640–1/1280) in all patients and IgG antibodies in most patients (see Fig. 3). Reactivity against GM2-positive melanoma cells and complement-mediated lysis was seen in over 90% of patients, and the antibody duration was 3–6 mo after each vaccination (61,63,64,73). Antibody titers have been maintained for over 3 yr by

Table 3
**Summary of Median Serological Results in Patients Vaccinated
With Monovalent Vaccines Against Carbohydrates**

Antigen	Total # of pts	ELISA			FACS			Median		CDC	
		% pts pos	IgM Pre/ post	IgG ^a Pre/ post	IgG Sub- class	% pts pos	IgM Pre/ post	IgG Pre/ Post	% pts pos	IA Pre/ post	% pts pos
GM2	12	100	0/640	0/320	IgG1+3	90	11/65	10/41	++	90	2/44
GD2L	12	80	0/320	0/160		60	10/38	11/11	50	0/30	
GD3L	12	70	0/40	0/160		50	9/30	10/30	+	40	2/54
FucGM1	18	100	0/320	0/320	IgG1	90	10/84	11/33	90	9/73	
Globo H	30	90	0/640	0/40	IgG1+3	75	10/41	10/13	++	55	4/36
Lewis Y	18	60	0/80	0		30	7/23	10/12	+	40	3/26
PolySA	6	100	0/640	0/20		80	10/48	10/12	0	- ^b	
Tn(c)	15	100	0/1280	0/1280		60	10/44	10/10	+	0	-
STn(c)	27	100	0/1280	0/160	IgG3	90	10/85	10/8	+	0	-
TF(c)	15	60	0/320	0/10		60	11/41	10/25	+	0	-

^a0 = titer less than 1/10.

^b- = not detected in any patient.

blank = not tested.

administration of repeated booster immunizations at 3- to 4-mo intervals. Antibody titers could not be further increased by pretreatment with a low dose of cyclophosphamide (300 mg/M²) to decrease suppressor-cell reactivity (*see* Fig. 3). As with the other carbohydrate antigen vaccines described below, no evidence of T-cell immunity detected by delayed-type hypersensitivity (DTH) skin test reactivity against GM2 was found.

This GM2-KLH plus QS-21 vaccine has been tested in a phase III randomized trial in melanoma patients in this country compared to high-dose interferon-alpha. The trial was stopped because after a median follow-up of 16 mo, patients receiving interferon had a significantly longer disease-free and overall survival. Longer follow-up will be required to determine the long-term impact, but the results to date indicate that induction of antibodies against GM2 in stage III melanoma patients is not associated with demonstrable benefit (73). This may be because whereas essentially all melanomas express some GM2, only a minority express enough GM2 to permit cell lysis with MAbs or immune sera.

Fucosyl GM1, like GM2, is highly immunogenic. Essentially all patients vaccinated with fucosyl GM1-KLH plus QS-21 produced IgM antibodies and most produced IgG antibodies (*see* Fig. 3) against fucosyl GM1 that also reacted with the SCLC cell surface by FACS and CDC (74).

Trials of GD2 and GD3 conjugated to KLH in melanoma patients induced only low (GD2) or no (GD3) antibodies reactive with the immunizing ganglioside or antigen-positive melanoma cells. GD2 and GD3 are clearly less immunogenic than GM2. Based on early work from Hakamori and colleagues (75), we have demonstrated that conversion of these two gangliosides to lactones by treatment with acid after conjugation to KLH

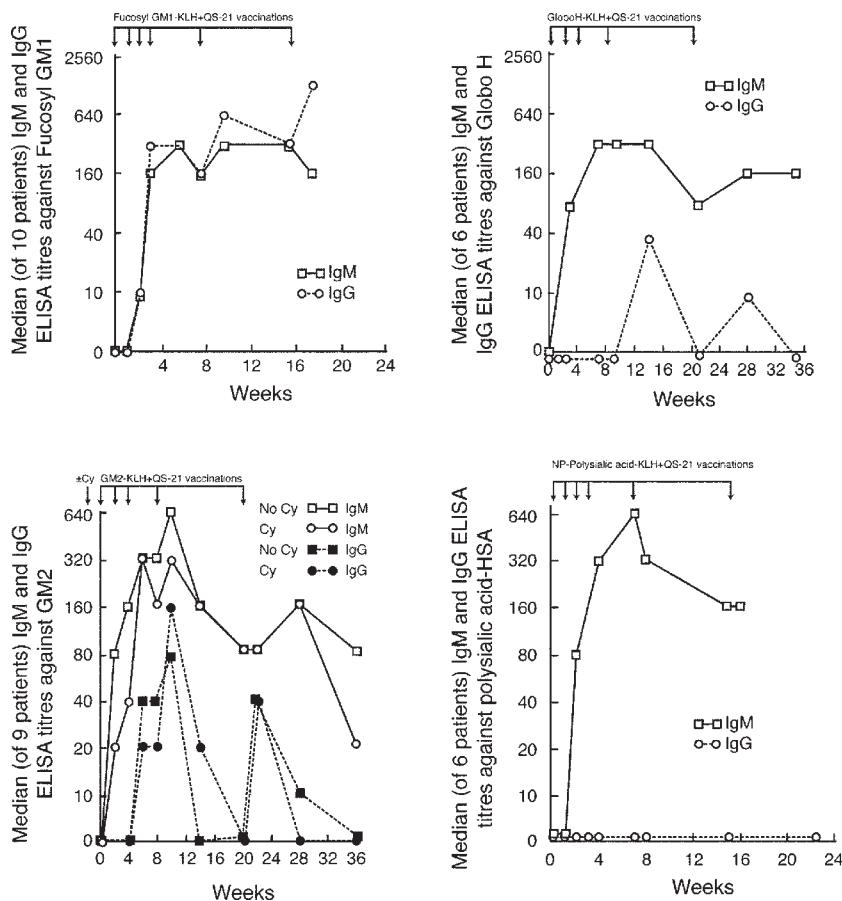


Fig. 3. Median sequential ELISA IgG and IgM antibody titers induced in groups of cancer patients after vaccination with KLH-carbohydrate antigen conjugate vaccines plus QS-21. Some patients receiving the GM2 vaccine were treated with 300 mg/M² cyclophosphamide before the first vaccination.

resulted in more immunogenic vaccines. Increased antibody titers against the native gangliosides and against tumor cells were induced in the majority of patients (see results in Table 3)(94).

3.3. *Le^y* and *Globo H* Vaccines

The development of *Le^y* and *Globo H* vaccines was previously limited by the lack of sufficient quantities of antigen for vaccine construction and testing. Over the last 6 yr, Dr. Samuel Danishefsky in our group has successfully synthesized both antigens (76–78). We have immunized groups of mice with *Globo H*-ceramide plus or minus adjuvants QS-21 and *Salmonella minnesota* mutant R595, and with *Globo H* covalently attached to KLH or bovine serum albumin (BSA) plus immunological adjuvants QS-21 or GPI-0100. The highest antibody titers against both synthetic antigen and MCF7 cells expressing *Globo H* were induced by the *Globo H*-KLH plus QS-21 vaccine (77). The antibody titer induced against synthetic *Globo H* was 1/120,000 by ELISA, the titer induced

against MCF7 was 1/320, and potent complement-mediated cytotoxicity was seen as well. Le^y-BSA and Le^y-KLH vaccines have also been tested in the mouse. High-titer antibody responses have resulted against the synthetic epitope of Le^y and against tumor cells expressing Le^y in the majority of mice immunized (80). Based on these results, clinical trials with Globo H-KLH plus QS-21 and Le^y-KLH plus QS21 have been initiated in patients with breast, prostate, or ovary cancer. The results are summarized in Table 3. Antibodies against the purified antigens and against tumor cells expressing these antigens were induced in most patients immunized with globo H (79; see Fig. 3) and occasional patients immunized with Le^y (81–83).

3.4. TF, Tn, and sTn Vaccines

Patients with various epithelial cancers have been immunized with unclustered TF-KLH and sTn-KLH vaccines plus various adjuvants (84,85). High-titer IgM and IgG antibodies against TF and sTn antigens resulted. In our hands the majority of the reactivity was against antigenic epitopes present in the vaccine that were not present on naturally expressed mucins (porcine or ovine submaxillary mucins [PSM or OSM]) or tumor cells (84,86). Based on previous studies with Tn antigen (87), Kurosaka and Nakada et al. hypothesized that MLS102, a monoclonal antibody against sTn, might preferentially recognize clusters ((c)) of sTn (88). Studies with monoclonal antibody B72.3 and with sera raised against TF-KLH and sTn-KLH conjugate vaccines in mice and in patients resulted in the same conclusion (41,84,86). The availability of synthetic TF, Tn, and sTn clusters consisting of three epitopes covalently linked to three consecutive serines or threonines has permitted proof of this hypothesis. In both direct tests and inhibition assays, B72.3 recognized sTn clusters exclusively, and sera from mice immunized with sTn (c)-KLH reacted strongly with both natural mucins and tumor cells expressing sTn (41). Based on this background, we initiated trials with the TF(c)-KLH, Tn(c)-KLH, and sTn(c)-KLH conjugate vaccines in patients with breast cancer. Antibodies of relevant high titer and specificity, including against OSM or PSM and cancer cells expressing TF, Tn, or sTn, were induced for the first time in our experience (Table 3). Based on these results, we plan to include clustered Tn, sTn, and TF in the polyvalent vaccines against epithelial cancers.

Several trials with TF, Tn, and sTn vaccines have been reported from other centers, and a large multicenter phase III trial with an sTn vaccine has been completed. George Springer's pioneering trials in breast cancer patients with vaccines containing TF and Tn purified from natural sources and mixed with typhoid vaccine (as adjuvant) began in the mid-1970s (34,89,90). DTH and IgM responses against the immunizing antigens and prolonged survival compared to historical controls were reported. MacLean immunized 10 ovarian cancer patients with synthetic TF conjugated to KLH plus immunological adjuvant Detox (monophosphoryl Lipid A plus BCG cell-wall skeletons) and described augmentation of IgG and IgM antibodies against synthetic TF in 9 of 10 patients (85). Lower levels of antibody reactivity against TF from natural sources were detected in some of these cases. MacLean has also immunized patients with breast and other adenocarcinomas with sTn-KLH plus Detox (13,51,91). Induction of IgM and IgG antibodies against synthetic and natural sources of sTn was seen in essentially all patients and this response was further increased by pretreatment of patients with a low dose of cyclophosphamide. Reactivity of these sera with natural mucins and tumor cells despite the use of an unclustered sTn vaccine is probably explained by the fourfold higher sTn/KLH epitope

ratio achieved in the MacLean vaccine compared to our previous unclustered vaccine. Survival appeared to be improved overall compared to historical controls and patients who responded with high antibody titers survived longer than those with lower titers. Reactivity with breast cancer cells, including CDC, was described. This is the basis for the completed multicenter phase III randomized trial of the sTn-KLH plus Detox vaccine vs KLH alone plus Detox in breast cancer patients with stable disease or clinical response to chemotherapy. Although the sponsor's website reports this study did not reach its primary endpoint, subgroup analysis is being performed.

3.5. Polysialic Acid Vaccines

Initial attempts at preparing a vaccine against polysialic acid for use in military recruits who are at risk of group B meningococcus infection were unsuccessful. We also have completed analysis of a clinical trial with polysialic acid conjugated to KLH plus QS-21 and found that no antibody response could be induced. Consequently, we tested a second polysialic acid vaccine that had been modified (*N*-propionylated) to increase its immunogenicity in collaboration with Dr. Harold Jennings, who pioneered the use of *N*-propionylation for this purpose (92). This induced an antibody response against unmodified polysialic acid in five of six patients immunized (see Fig. 3). These vaccine-induced antibodies also reacted with SCLC cells (and were cytotoxic for antigen-positive bacteria). This *N*-propionylated polysialic acid vaccine is suitable for inclusion in our polyvalent vaccine against SCLC and possibly for trials in students and military recruits for prevention of group B meningococcus infections.

4. EFFECTOR MECHANISMS OF ANTIBODIES AGAINST CELL-SURFACE ANTIGENS

Immunization against the carbohydrate components generally results exclusively in an antibody response (see 15–17 for dissenting views), primarily an IgM antibody response. These IgM antibodies are known to induce complement activation resulting in inflammation, and phagocytosis of tumor cells by the reticuloendothelial system (opsonization) and CDC (reviewed in 1). IgG antibody responses can also induce complement activation (regarding IgG depending on the subclass, IgG1 and IgG3 being optimal in humans), and these same effector mechanisms. IgG antibodies of these subclasses are also known to induce ADCC. Serological analysis of the series of clinical trials described above has suggested that the six vaccines containing different glycolipids induced antibodies mediating CDC whereas the four vaccines containing carbohydrate or peptide epitopes carried by mucin molecules induced antibodies that were not capable of mediating CDC. To determine whether this dichotomy was a result of the properties of the induced antibodies (i.e., class and effector functions), the different target cells used, or the nature of the target antigens, we compared the cell-surface reactivity (assayed by FACS), complement-fixing ability (using the immune adherence [IA] assay), and the CDC activity of a panel of monoclonal antibodies and immune sera from these trials on the same two tumor cell lines. Antibodies against glycolipids GM2, globo H and Le^y, protein KSA, and mucin antigens Tn, sTn, TF, and MUC1 all reacted with these antigens expressed on tumor cells and all fixed complement. CDC, however, was mediated by antibodies against the glycolipids and a globular protein (KSA), but not by antibodies against the mucin antigens.

It must be emphasized that although we showed that mucins are poor targets for complement-mediated lysis of tumor cells, studies have shown that induction of antibodies against either glycolipid or mucin antigens results in protection from tumor recurrence in several different preclinical mouse models (reviewed in refs. 8 and 9). Also, antibodies against either glycolipid or mucin epitopes correlate with a more favorable prognosis in patients (11–13,90). It does not appear that the inability of antibodies against mucin antigens to induce complement-mediated lysis is necessarily detrimental to the antitumor response. Consequently, complement-mediated inflammation, opsonization, and ADCC but not CDC are likely mechanisms for the prolonged survival seen in the preclinical experiments targeting mucin antigens and suggested in the clinical trials with passively administered and actively induced antibodies against mucin antigens. Regarding bacterial infections, this is supported by the severe consequences of hereditary deficiency states involving either the classical or alternate complement pathways and the comparatively trivial consequences to deficiencies of the complement membrane attack complex (93).

5. SUMMARY

The majority of even cancer patients who will eventually die of their cancer can initially be rendered free of detectable disease by surgery and/or chemotherapy. Adjuvant chemotherapy or radiation therapy at this point are generally only minimally beneficial, so there is real need for additional methods of eliminating residual circulating cancer cells and micrometastases. This is the ideal setting for treatment with a cancer vaccine. The immune response induced is critically dependent on both vaccine design and awareness of the antigenic epitope. For antibody induction there is one best vaccine design, conjugation of the antigen to an immunogenic protein such as KLH and the use of a potent adjuvant such as the saponins QS-21 and GPI-0100. This approach alone induced strong antibody responses against the glycolipids GM2, fucosyl GM1 and globo H, and cancer cells expressing these glycolipids. Other carbohydrate antigens require additional modifications to augment relevant immunogenicity. GD2 and GD3 lactones and N-propionylated polysialic acid were significantly more effective at inducing antibodies against the unmodified antigens and tumor cells expressing these antigens. Tn, sTn, and TF trimers (clusters) were significantly more effective than the monomers at inducing antibodies reactive with the cancer-cell surface.

Antibodies are ideally suited for eradicating pathogens from the bloodstream and from early tissue invasion. Passively administered and vaccine-induced antibodies have accomplished this, eliminating circulating tumor cells and systemic or intraperitoneal micrometastases in a variety of preclinical models, so antibody-inducing vaccines offer real promise in the adjuvant setting. Polyvalent vaccines will probably be required because of tumor cell heterogeneity, heterogeneity of the human immune response, and the correlation between overall antibody titer against tumor cells and antibody effector mechanisms. Over the next several years, phase II clinical trials designed to determine the clinical impact of polyvalent conjugate vaccines will be initiated in the adjuvant setting in patients with SCLC and several epithelial cancers.

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12

Anti-Idiotype Vaccines

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1. INTRODUCTION

Active specific immunotherapy (ASI) is an attractive approach to cancer therapy, especially in an adjuvant setting. ASI is intended to boost or induce a host antitumor response, in contrast to passive immunotherapy, where large doses of preformed antitumor antibodies, or T cells with predetermined specificity, are infused. In classical ASI, patients are vaccinated with purified tumor-specific or tumor-associated antigens (TAAs). This approach has a number of major limitations. The tumor antigens are usually weakly immunogenic due to the induction of tolerance. This tolerance can be broken by presentation of the critical epitope in a different molecular environment (1). Secondly, it is difficult to obtain the purified antigen in sufficient quantities for vaccination. Although this limitation can be overcome by the synthesis of well-defined antigens by use of recombinant DNA technology, the recombinant molecule may not resemble the native structure of the protein. Moreover, mass production of nonprotein antigens, such as carbohydrates or lipids, is not possible by recombinant DNA technology. These limitations can be overcome by using an elegant approach to ASI, with the use of anti-idiotypic (Id) antibodies.

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Anti-Id antibody approach is based on the internal-image concept (2,3). This concept proposes that the idiotypes represent links between the outside world of the antigens and the inner immune repertoire. The antigen receptor of T and B lymphocytes expresses antigenic determinants that can be recognized and can elicit humoral or cellular immune responses. Anti-Id concept for the induction of humoral responses can be summarized as follows. A given antibody, Ab1, can recognize a specific determinant on an antigen (Fig. 1A), such as a tumor antigen. The interaction of Ab1 to the antigenic epitope takes place through its variable regions. The variable regions of the Ab1 can also serve as a determinant that induces the synthesis of a heterogeneous population of antibodies, referred to as Ab2 or anti-Id antibodies (Fig. 1B). There can be three classes of Ab2 antibodies based on the region of the variable domain of Ab1 they recognize (4). Ab 2α recognize idiotopes that are outside the antigen-binding site. If the target idiotope is close to the binding site and interferes with the antigen binding, it is called Ab 2γ . Ab 2β recognize the binding site of Ab1 and resemble the epitope recognized by Ab1 (Fig. 1B). Ab 2β , thus, can act as a surrogate for the nominal antigen and can be used as vaccines. Ab 2β antibody is defined by three criteria (5):

1. Immunochemical criterion: its ability to block the binding of Ab1 to its antigen. Antigen binding by Ab1 is not affected by Ab 2α ; may be partially blocked by Ab 2γ .
2. Functional criterion: The functional criterion is based on its ability to mimic a given antigen and, therefore, to induce the synthesis of antibody specific for the same antigen in various species (genetically unrestricted). Immunization of an animal with Ab 2β elicits a polyclonal anti-anti-Id antibody or Ab3 response (Fig. 1B). A subset of Ab3 is expected to recognize the nominal antigen. This Ab3 subtype is called Ab1' to indicate that it might differ in its other idiotopes from Ab1 (Fig. 1B).
3. Structural criterion: Structural identity between an epitope of the antigen and a segment of the variable region of the antibody may represent the most faithful criterion to define an Ab 2β .

Whereas the structural criterion may be the best for designation of Ab 2β raised by immunization with antibodies specific for protein antigens, the immunochemical and functional criteria can be used to define Ab 2β raised against antibodies specific for polysaccharides, lipoproteins, nucleotides, or synthetic drugs (5). The cascade of complementary idiotopes is the basis of making anti-Id antibody vaccines for cancer and a number of infectious diseases (5). The efficacy of anti-Id vaccination has been demonstrated in a number of murine tumor models as well as in cancer patients.

2. ANTI-ID ANTIBODIES IN MURINE TUMOR MODELS

In a number of studies, Kennedy et al. (6,7) demonstrated the efficacy of anti-Id vaccines in a model of SV40-transformed tumor cells. An anti-Id antibody, 58D, was raised against a monoclonal antibody, PAb 405. Pab 405 recognizes the carboxy terminus of SV40 T-antigen. Immunization of BALB/c mice with 58D induced high-titer anti-T-antigen antibodies (Ab1') in 6 of 10 mice. Three of the mice with the highest anti-T-antigen titers were completely protected against challenge with lethal doses of live SV40-transformed mKSA tumor cells. Nepom et al. (8) raised anti-Id antibodies against murine monoclonal antibody 8.2, an antibody specific for a human melanoma-associated cell-surface marker called p97. They demonstrated that these anti-Id antibodies can

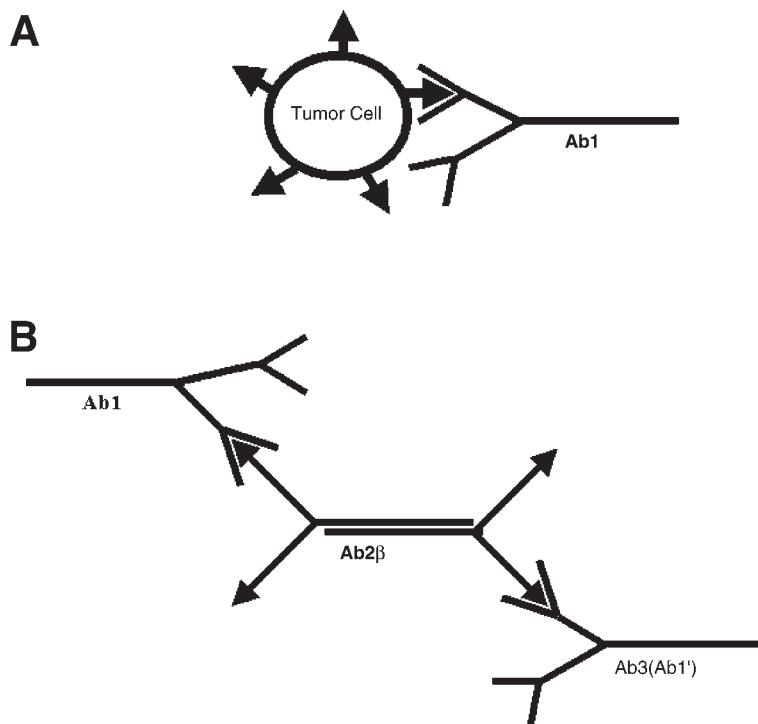


Fig. 1. Idiotype network. (A) Binding of monoclonal antibody to an epitope of a tumor antigen, ↑. (B) Generation of $\text{Ab}2\beta$, $\text{Ab}3$ and $\text{Ab}1'$. See text for details.

prime animals for immunity with specificity that mimics 8.2 itself. Sera from mice immunized with anti-8.2 (Ab2) contained anti-anti-8.2 antibodies (Ab3), some of which have the same idiotype as 8.2 itself (Ab1'). In another model, Raychaudhuri et al. (9) generated a number of anti-Id antibodies using 11C1 as the Ab1 antibody. 11C1 recognizes the gp52 envelope of mouse mammary tumor virus. Immunizing DBA/2 mice with monoclonal anti-Id antibodies induced immune responses related to gp52. Dunn et al. (10) studied the anti-Id cascade in a rat sarcoma model, HSN. The Ab2 antibody (HIM/1/230) was developed using the Ab1 11/160, a monoclonal antibody against HSN tumor cells. Tumor progression was evaluated by a lung colonization assay. Compared to untreated mice, those treated with this Ab2 had significantly reduced number of surface colonies following challenge with intravenous injection of rat sarcoma HSN tumor cells. Anti-Id antibody prepared against anti-BCG (bacille Calmette-Guérin) monoclonal antibody (11) exhibited vaccine activity against Meth A fibrosarcoma that shared a common antigen(s) with BCG. Mice vaccinated with the anti-Id antibody were partially protected against Meth A tumor, and the presence of Ab3 type antibody was detected in the sera of immunized mice. To determine whether the pulmonary metastases of melanoma cells could be inhibited by anti-Id therapy, C57BL/6 mice were immunized with an anti-Id antibody, 7C4, which mimics a mouse melanoma antigen. Vaccination of mice with 7C4 significantly reduced lung metastasis ($p < 0.01$) and increased survival ($p < 0.01$) following challenge with live BL6 cells into their caudal (12). An anti-Id monoclonal

antibody, D704, was established that bore the internal image of the determinant defined by the monoclonal antibody, M2590, against the sialic acid residue on ganglioside, GM3. Significant suppression of tumor growth and prolongation of survival were achieved by immunization with D704 followed by challenge with 1×10^4 melanoma cells per mouse, but not in a group inoculated with 5×10^4 melanoma cells/mouse. D704 induced humoral anti-anti-Id (anti-GM3) response as well as cellular antitumor responses (13).

We developed a murine monoclonal anti-Id antibody, designated 3H1, which mimics a specific epitope of carcinoembryonic antigen (CEA) (see Subheading 2.1.1. for details). The efficacy of 3H1 as a tumor vaccine was evaluated in a murine model (14). In this model, the murine colorectal cancer cell line MC-38 was transduced with human CEA gene and injected into syngeneic C57BL/6 mice. Immunization of naïve mice with 3H1 induced humoral and cellular anti-3H1 as well as anti-CEA immunity. Mice immunized with 3H1 were protected against a challenge with lethal doses of MC-38cea cells, whereas no protection was observed with CEA-negative MC-38 cells, or when mice were vaccinated with an unrelated anti-Id antibody and challenged with MC-38cea cells ($p < 0.003$). To demonstrate the efficacy of 3H1 vaccine against established tumors, MC38cea tumors were first implanted into the mice and they were treated with 3H1 or a control anti-Id antibody. At first tumors developed in both groups at the same rate. On completion of six courses of treatment, tumors of six of nine mice treated with 3H1 became necrotic and regressed. In the control group, only one of eight mice showed regression. These data demonstrate that 3H1 induced CEA-specific antitumor protective immunity in this murine model (14).

Overall, these results in murine systems suggest that anti-Id vaccines bearing internal images of protein and nonprotein antigens induce humoral and cellular immunities directed against the nominal antigens. The vaccinated mice are protected against subsequent challenge with live tumor cells. These results stimulated interest in the development of anti-Id vaccines for the treatment of cancer patients.

3. ANTI-ID ANTIBODIES AND HUMAN CANCER

3.1. Colorectal Cancer

3.1.1. CARCINOEMBRYONIC ANTIGEN (CEA)

A number of anti-Id antibodies (Table 1) have been generated that mimic CEA (15–23). CEA is one of the first TAAs to be identified and is one of the most widely investigated human TAAs. CEA is a 180 kD glycoprotein and was originally detected in colonic carcinoma and fetal gut. However, it has since been detected at high density in 95% of colorectal carcinoma, 70% of lung adenocarcinoma, and 50% of breast cancer. In order to generate the most highly specific and the least cross-reactive Ab1' antibodies, a suitable anti-CEA monoclonal antibody should be chosen. A number of anti-CEA monoclonal antibodies have been raised, some of which react with the carbohydrate moiety of the molecule, whereas others recognize the peptide part, which may, however, be present in CEA-related antigens. Such CEA-related antigens include the normal cross-reacting antigens (NCAs), NCA2, normal fecal antigen (NFA-2), and biliary glycoprotein (BGP), all of which are also present in a number of normal tissues. For the generation of the anti-Id antibody 3H1, mimicking CEA, we selected a monoclonal anti-CEA antibody, 116NS-3d or 8019, which recognizes a high molecular weight CEA primarily associated with human colonic and pancreatic carcinoma. 3H1 mimics a unique epitope

Table 1
Anti-Idiotype Antibodies Mimicking Human Tumor-Associated Antigens

Cancer	TAA	Ab1	Ab2	References
Colorectal	CEA	8019	3H1, IgG1κ	15
	CEA	NCRC23	708, IgG2b	21
	CEA	NP-3	CM1, IgG1	16
	CEA	NP4	CM11, IgG1	16
	CEA	NP4	Polyclonal ^a	17
	CEA	MN14	W12, IgG1 ^b	19
	CEA	T84.66	Mald6G6.C4	23
	CO17-1A	CO17-1A	Polyclonal ^c	27
	CO17-1A	CO17-1A	BR3E4 ^b	28
	GA733-2	17-1A	h-Ab2 ^d , IgG1κ	29
Melanoma	791gp72	791T/36	105AD7, IgG1 ^d	30
	HMW-MAA	225.28	MF11-30, IgG1	33
	HMW-MAA	763.74	MK2-23, IgG1	34
	MPG	MEM-136	I-Mel, IgG1	35
	GD2	14G2a	1A7, IgG1κ	38
	GD3	R24	BEC2, IgG2b	39
Ovarian	GM3	M2590	D704, IgG1	13
	GM3	L612	4C10, IgG1κ	41
	CA 125	OC 125	ACA 125, IgG1κ	42
Breast	TAG-12	12H12	5H8, IgG1κ	44
	HMFG	MC-10	11D10, IgG1κ	45
Lymphoma/ Leukemia	Her2/neu	520C9	520C9-6b, IgG1κ	46
	IRac	antiIRac	4B4, IgG1κ	49
Leukemia	Gp37	SN2	4DC6, IgG1κ	51

Note: All anti-idiotype antibodies were raised in mice, except in ^ababoon, ^brat, ^cgoat, ^dhuman.

on CEA, which is not present on other CEA-related molecules. 3H1 induced CEA-specific antibodies in mice, rabbits, monkeys, and humans. These immune sera competed with the Ab1 antibody for binding to CEA-positive colon carcinoma cell line LS174T, and inhibited binding of radiolabeled Ab1 to Ab2, suggesting that the induced Ab3 share idiotopes with Ab1. Furthermore, monoclonal Ab3 that bind to CEA was generated from mice immunized with 3H1. The Ab3 (both polyclonal as well as monoclonal) immunoprecipitated the same 180 kD CEA as the Ab1 antibody by Western blot analysis, and showed almost identical immuno-staining patterns as Ab1 on colonic adenocarcinoma tissue sections from several patients (15). Variable domains of 3H1 showed sequence homology to CEA (24), suggesting that 3H1 meets the structural criterion of an Ab2β antibody (5).

We have completed phase I/II clinical trials with 3H1 as immunogen in advanced colorectal cancer patients (reviewed in ref. 25). The results of these studies appear to be promising and 3H1 is currently being tested in a phase III trial in patients with resected colon carcinoma. Goldenberg's group generated a number of Ab2 antibodies mimicking various CEA epitopes. These Ab2 were raised in mice (16), rat (19), baboon (17), as well as in cancer patients (18). Their data indicated that in each case the Ab2 molecule exhibits

the immunochemical features of an internal-image antibody mimicking a distinct epitope on CEA. Durrant and her group (21) generated an anti-Id, 708, by immunization of mice with anti-CEA monoclonal antibody NCRC23. Although the anti-Id antibodies mimicking CEA, listed in Table 1, appear to have clinical potential as network antigen for active immune therapy of patients with CEA-positive tumors, to the best of our knowledge, none except 3H1 has yet been tested in clinical trials.

3.1.2. CO17-1A/GA733 ANTIGEN

CO17-1A/GA733 is a nonsecreted 40-kD glycoprotein TAA. Although it is present in trace quantities in a number of normal tissues, the majority of tumor cells in almost all colorectal cancer patients express this antigen in high density. This antigen is defined by the monoclonal antibodies, 17-1A and GA733, which bind to different epitopes on the antigen. CO17-1A/GA733 has adhesion functions; therefore, it is also designated as an epithelial cell adhesion molecule (Ep-CAM). It has also been designated as EGP, KSI-4, and KSA (26). A polyclonal Ab2 was prepared in goat using the monoclonal 17-1A antibody. Clinical trials with this polyclonal anti-Id induced partial remission in a number of colorectal cancer patients (27). Recently a monoclonal anti-Id antibody in rat, BR3E4, mimicking the CO17-1A antigen was used for clinical trial (28). Ab3 as well as Ab1' were induced in the sera of a number of cancer patients in this trial. Fagerberg et al. (29) generated one Ab2 β , h-Ab2 from EBV-immortalized B cells from a colorectal cancer patient treated with the monoclonal Ab1 molecule, 17-1A. Induction of Ab1' antibodies in the treated patients were observed in five of six patients.

3.1.3. 791GP72/CD55

Anti-Id antibody 105AD7 is a human monoclonal antibody produced by fusion of a mouse/human heteromyeloma cell line with lymphocytes from a patient previously injected with mouse monoclonal antibody 791T/36 (30). 791T/36 is directed against the antigen 791gp72, which has recently been shown to be CD55, a protein involved in regulating activated complement component at the cell surface. It is present at low levels on all cells that are exposed to complement and has been shown to be upregulated 40- to 100-fold (31) in a number of solid tumors including colorectal cancer. Immunization of mice and rats with 105AD7 resulted in the generation of Ab3 antibodies that bind CD55 antigen, suggesting that 105AD7 is an Ab2 antibody mimicking CD55. Clinical studies with 105AD7 showed improved survival of the treated patients when compared to control groups, although the data were not statistically significant (32).

3.2. Melanoma

A number of murine monoclonal antibodies directed against tumor-associated melanoma antigens have been used for the generation of anti-Id antibody. Some of these TAAs are glycolipids.

3.2.1. HIGH-MOLECULAR-WEIGHT-MELANOMA-ASSOCIATED PROTEIN (HMW-MAA)/MPG

HMW-MAA, alternatively designated as MPG, is a high-molecular-weight (>400 kD) chondroitin sulfate proteoglycan present in high density on more than 80% of cutaneous melanoma with limited heterogeneity. It is less frequently present in ocular melanomas. It is present on metastases as well as primary lesions. The distribution of this antigen is

also restricted in normal tissues. All these properties make HMW-MAA a suitable target for immunotherapy.

Mittelman et al. (33) generated an anti-Id antibody, MF11-30, which mimics HMW-MAA by injection of the monoclonal antibody 225.28. In clinical trials with this anti-Id, minor responses were observed in a few patients. A second anti-Id antibody, MK2-23, was developed using the monoclonal anti-HMW-MAA antibody, 763.74, as the Ab1 antibody (34). About 50% of the patients developed Ab1' antibodies in this trial. Partial responses were observed in a few patients. Survival of the patients who developed Ab1' type antibody was significantly ($p<0.0003$) longer than those without detectable Ab1' type antibodies. Another group of investigators (35) used a different anti-Id antibody, I-Mel-2 (MELIMMUNE-2), for immunotherapy of metastatic melanoma. I-Mel-2 was generated by immunization of mice with anti-HMW-MAA monoclonal antibody MEM-136 as the Ab1. MEM-136 recognizes a different epitope of HMW-MAA than those recognized by antibodies 763.74 and 225.28. I-Mel-2 along with an adjuvant, SAF-m, was administered to 26 patients, 6 of whom showed positive clinical responses.

3.2.2. DISIALOGANGLIOSIDE (GD2)

Increased and aberrant membrane expression of different gangliosides, such as GD2, on tumors of neuroectodermal origin including malignant melanoma, neuroblastoma, soft-tissue sarcoma, and small-cell carcinoma of lung tumor, has been reported (36). GD2 is absent in most normal tissues, except for low levels in brain and peripheral nerve. Thus, GD2 is a suitable target for immunotherapy of tumors of neuroectodermal origin. Although vaccination of cancer patients with different gangliosides has been tested by a number of investigators, GD2 is weakly immunogenic in humans (37). For the preparation of effective GD2 vaccines, the ganglioside has to be conjugated to keyhole limpet hemocyanin (KLH) and administered along with a strong adjuvant. Moreover, complex purification steps are involved for the preparation of purified GD2. These limitations of GD2-based vaccines can be overcome by using the anti-Id approach.

We have generated anti-Id antibody, 1A7, by using the anti-GD2 monoclonal antibody, 14G2a, as the Ab1 antibody (38). Biological and serological assays established that 1A7 mimics GD2. We have initiated a phase Ib clinical trial for advanced melanoma patients. Out of 47 patients treated, sera from 40 contained Ab1' antibodies, which were predominantly IgG1. Some of these patients showed partial responses (25).

3.2.3. GANGLIOSIDE (GD3)

Virtually all melanoma tumors express abundant amounts of GD3, although it is present in small quantities in a number of normal tissues. GD3 appears to play a role in melanoma cell adhesion to solid substrates and antibodies against GD3 inhibit melanoma cell attachment, a process that is crucial in metastasis. Thus, GD3 is of particular interest as a target for immunotherapy. GD3, however, proved to be a weak immunogen in humans (37). Because of this, Chapman and his group decided to adopt the anti-Id antibody approach based on GD2 for the development of ASI. Anti-Id antibody, BEC2 was raised against the anti-GD3 monoclonal antibody R24 (39). Initial trials with melanoma patients failed to show development of immunity. Use of BEC2 conjugated to KLH along with BCG as adjuvant induced IgM anti-GD3 antibodies and enhanced survival was noticed in a number of patients (40).

3.2.4. GANGLIOSIDE (GM3)

Ganglioside GM3 also occurs in high density on melanoma cells. Two anti-Id antibodies mimicking GM3 have been developed (13,41). The characteristics of these anti-Id antibodies suggest that these are internal-image antigens. These antibodies, however, have not been tested for clinical applications.

3.3. Ovarian Cancer

3.3.1. CA 125

CA 125 is a high molecular weight (>500 kD) glycoprotein present on most of the nonmucinous epithelial ovarian tumors. Antigenic determinants of CA 125 complex are found in celomic epithelium during embryonic development and can be detected on fetal tissues, Mullerian duct remnants in the genital tract, on amnion, and in amniotic fluids. As a result, cancer patients are immunologically tolerant to this self-antigen. Since anti-Id antibodies have been demonstrated to break tolerance to self-antigens, ASI using this approach is suitable for CA 125 positive cancer patients. Schlebusch et al. (42) generated a murine monoclonal anti-Id antibody, designated ACA 125, bearing the internal image of CA 125. Recently a clinical trial of advanced ovarian cancer patients with ACA 125 (43) was carried out. Hyperimmune sera of 28 of 42 patients had variable levels of Ab3, predominantly immunoglobulin G (IgG) isotype. Survival of patients in whom Ab3 was induced by vaccination was significantly ($p<0.0001$) longer than Ab3 negative patients.

3.3.2. TAG-12

Tumor-associated glycoprotein 12 (TAG-12) is a high molecular weight (>200 kD) mucinous glycoprotein and is strongly expressed in about 96% of breast cancer patients and nearly 68% of ovarian cancer. TAG-12 is secreted in the sera of patients and can be used as a tumor marker. Schmitt et al. (44) produced 16 anti-Id antibodies by injection of the mono-clonal anti-TAG-12 antibody 12H12 into BALB/c mice. Two of these anti-Id antibodies, 5H8 and 5H2, were purified for further characterization. Immunization of rabbits with 5H8 induced Ab3, and part of the Ab3 behaved like Ab1'.

3.4. Breast Cancer

3.4.1. HUMAN MILK-FAT GLOBULE (HMFG) ANTIGEN

HMFG is a high molecular weight (>400,000 kD) mucin-like glycoprotein present in minute amounts in normal human mammary epithelial cells and is increased by at least 10-fold on breast carcinoma cells. HMFG is present in over 90% of breast tumor samples and is considered a high-profile target for immunotherapy of breast cancer. We have raised an anti-Id antibody, 11D10, against the monoclonal anti-HMFG antibody MC-10 (45). Serological and immunological characterization demonstrated that 11D10 is an internal-image antigen. A segment of the variable region of 11D10 showed sequence homology to a part of the HMFG molecule. Thus, 11D10 is an Ab 2β antibody also by structural criterion. We have completed a phase Ib clinical trial for patients with advanced breast cancer with 11D10 and most of the patients treated with 11D10 developed Ab1' antibodies in the sera (25).

3.4.2. HER2/NEU

Her2/neu, also known as c-erbB-2, is a 185 kD transmembrane protein that is overexpressed in high density on the surface of a number of human cancers. It is a member of the epidermal growth factor receptor family and is presumed to function as a growth factor receptor. Her2/neu is overexpressed in 25–30% of human breast cancer specimens. In normal cells Her2/neu gene is present as a single copy. Amplification of Her2/neu gene and/or overexpression of the associated protein has been directly related to poor prognosis of cancer patients. Extensive work in animal models has indicated that blocking of Her2/neu receptor with monoclonal antibodies can reduce the rate of tumor growth. These studies led to several clinical trials with humanized anti-Her2/neu monoclonal antibodies. Her2/neu, however, is a nonmutated self-protein, which limits its use in ASI owing to development of immune tolerance. Under these situations, anti-Id approach to ASI using Her2/neu as target molecule should be undertaken. We have generated an anti-Id antibody, 520C9-6b, by using murine monoclonal anti-Her2/neu antibody, 520C9 (46). Immunization of small animals induced Ab1' type antibodies in the sera. As yet, we have not used 520C9-6b for clinical trials.

4. NETWORK ANTIGENS IN LYMPHOMA AND LEUKEMIA

Apart from solid tumors, anti-Id antibodies have been developed for the treatment of T- and B-cell lymphoma.

4.1. IRac Antigen

IRac is a 70 kD protein present on the surface of Hodgkin-Reed-Sternberg and interdigitating reticulum cells. IRac is rarely detected in normal or antigen- or mitogen-activated lymphocytes, but is observed frequently in virus-transformed B or T lymphocytes. The antigen is absent from cells in most non-Hodgkin's lymphomas and is a suitable target for immunotherapy (47). By injection of anti-IRac antibody as Ab1, Schobert et al. (48) generated an anti-Id antibody 4B4. Serological characterization of 4B4 suggested that it is a network antibody. 4B4 induced IRac specific Ab3 in mice and New Zealand white rabbits. BALB/c mice immunized with 4B4 showed DTH reaction against IRac-expressing Hodgkin's cell lines. 4B4 has the potential for use in the treatment of Hodgkin's lymphoma, although no clinical study using 4B4 has been published.

4.2. Gp37 Antigen

Glycoprotein Gp37 is found on 70% of children with T-cell acute lymphoblastic leukemia and 30% of T-cell lymphoma, but is absent on normal or activated T-lymphocytes or other hematopoietic or nonhematopoietic tissues. We generated an anti-Id antibody, 4DC6, mimicking gp37 and established that 4DC6 is an Ab2β type antibody (49). 4DC6 was tested in a phase I clinical trial involving four patients with T-cell lymphoma primarily confined to the skin (50). In three of four patients, immunization with 4DC6 induced Ab3. The Ab3 antibody of one patient bound specifically to Gp37 antigen-positive MOLT-4 cells and to semipurified Gp37 antigen, suggesting the induction of Ab1' antibody in this patient. This patient, who had nine discrete skin tumors and peripheral blood involvement without other detectable disease, had virtually complete disappearance of the tumors, lasting over 11 mo. Tumors in this patient regrew despite 4DC6 therapy, however, those tumors were Gp37-negative (50).

5. FORMATS OF ANTI-ID ANTIBODIES

5.1. Single-Chain Anti-Id Antibodies

5.1.1. 3H1 (CEA Mimic)

Since variable regions are unique for an antibody molecule and define its reactivity, we sought to determine whether the single chain-variable fragments (scFv) of an anti-Id is adequate for its antigen mimicry. We chose scFv format of 3H1 for this purpose. In scFv the carboxy terminus of one variable domain (variable heavy, VH, or variable light, VL) is joined to the amino terminus of the second variable domain by a linker (Ln) of appropriate length and flexibility. The scFv can be constructed with the sequence VH-Ln-VL or VL-Ln-VH. The VH-VL construct typically exhibits Euclidean distances between Ln termini of 29–35 Å, whereas the VL-VH orientation generally exhibits distances that are 5–10 Å longer for the same scFv (51). Thus, different orientation of domain may distort the native scFv conformation. Distortion of the scFv derived from an anti-Id antibody is likely to affect its antigen mimicry. We therefore made four different formats of scFv. In two formats VH-Ln-VL and VL-Ln-VH, VH and VL were linked by a 15 amino acid Ln (Gly₄Ser)₃. In VH-VL and VL-VH, Ln was omitted (52). Comparison of the binding of the Ab1 antibody, 8019, (15) to purified scFv proteins by ELISA and immunoblot experiments, showed that only VH-Ln-VL had significant activities in this assays. VH-Ln-VL also showed maximum inhibition of binding of 8019 to CEA. Immunization of mice with naked VH-Ln-VL and VH-Ln-VL or conjugated to KLH, induced anti-CEA antibodies in the immunized mice sera. Induction of anti-CEA antibodies in the immunized mice was confirmed by flow cytometric analysis using CEA-positive tumor cell lines. Isotypic analysis of the Ab3 (Ab1') of the immunized mice, however, demonstrated that the majority of these antibodies are IgM.

5.1.2. ACA125 (CA125 Mimic)

ScFv format of the anti-Id antibody, ACA125, was engineered essentially as described for 3H1. Rats were immunized with this scFv mixed with Freund's adjuvants. Ab3 as well as Ab1' antibodies were induced in the immunized rats. However, in contrast to 3H1 scFv, the antibody titers were lower with ACA125 scFv immunogen when compared to the whole molecule (53).

5.2. Anti-Id DNA Vaccines

Our anti-Id antibody, 1A7 mimicking GD2, induced both Ab3 and Ab1' humoral responses in injected small animals (54), primates (38), as well as melanoma patients (55). However, presumed cellular responses could not be detected in the injected patients. Various plasmid constructs have been reported to invoke combined humoral and cellular immune responses in injected hosts against the transgene product. To investigate the induction of cellular immune responses we constructed two plasmids expressing 1A7 scFv for use as vaccines. One of the plasmids expressed the VH-Ln-VL format of scFv, whereas the other expressed the VL-Ln-VH format (56). Following intramuscular injection of mice, the plasmids were detectable in the injected tissues for at least 3 mo and the injected plasmids actively transcribed the scFv of 1A7 at the injected site. A single intramuscular immunization of mice with either of the plasmids in phosphate-buffered saline induced humoral immune responses against 1A7 as well as GD2. Multiple immunizations, however, were required to elicit stronger humoral responses. No cellular

responses were invoked by immunization with the plasmids (56). Thus, for anti-Id antibody therapy, the DNA vaccines do not appear to be better than the protein vaccines.

6. IMMUNE PATHWAYS INDUCED BY ANTI-IDIOTYPIC ANTIBODIES

6.1. *Antibody-Mediated Immunity*

Except in a few instances (57), Ab1' antibodies were detectable in the sera of cancer patients and animals injected with anti-Id antibodies. The Ab1' generated can eradicate tumor cells by antibody-dependent cellular cytotoxicity (ADCC), cell-mediated cytotoxicity (CMC), or induction of apoptosis. Ab1' antibodies generated in mice by immunization with the anti-Id antibody 3H1 were cytolytic for CEA-positive tumor cell by ADCC assays (14). No lysis was detected with the CEA-negative tumor cells in these assays. Although the extent of specific cell lysis by sera from each individual mouse varied, significant ADCC was observed in all the injected mice. Sera from 11 out of the 17 colorectal cancer patients vaccinated with 3H1 showed ADCC. Interestingly, samples from patients whose sera mediated ADCC always contained anti-CEA antibodies (58). Schlebush et al. (42) demonstrated that sera from rats immunized with the anti-Id antibody ACA 125, induced antigen-specific ADCC as well as complement-dependent cytotoxicity (CDC).

6.2. *DTH and Helper T-Cell-Mediated Immunity*

Initiation of anti-Id cascade is T-cell dependent, since T-cell-deficient mice do not produce anti-Id antibodies upon immunization (59). Moreover, Ab3 antibody population induced by an anti-Id antibody predominantly contains IgG, suggesting the activation of helper T cells mediated by the anti-Id antibodies (60). Generation of idiotypic peptide in endoplasmic reticulum (ER) has been demonstrated by Weiss and Bogen (61), suggesting that CD4+ T cells recognize idiotypic peptides generated via endogenous pathway, rather than in the endosomal compartment following the internalization of the immunoglobulin molecule (5).

Anti-Id antibodies stimulated delayed-type hypersensitivity (DTH) responses toward the nominal antigen following immunization of small animals with the Ab2 antibody (8,9,13,62–65). A number of cancer patients treated with Ab2 showed DTH responses toward the Ab2 as well as the nominal antigen (29,66,67).

Generation of helper T cells has been evaluated by in vitro T-cell proliferation assays. Splenocytes isolated from non-tumor-bearing mice immunized with 3H1 were stimulated in the presence of 3H1, as well as the nominal antigen CEA. No stimulation was observed with splenocytes from naïve mice and at least three to four immunizations were needed to detect the stimulation (14). Peripheral blood mononuclear cells (PBMC) isolated from cynomolgus monkeys showed T-cell stimulation in the presence of the anti-Id antibody 11D10. No stimulation could be observed using isotype-matched antibodies (68). Increased T-cell proliferation, following anti-Id vaccination, has been also observed in cancer patients (21,22,24,29,50,58,67,69). In the clinical trial of postsurgical colorectal cancer patients (70), using the anti-Id antibody 3H1, 3H1 consistently generated potent anti-CEA cellular responses detected by the T-cell proliferation assays.

To study the cellular immunity invoked by 3H1 at the molecular level, we cloned and sequenced the cDNA, encoding the VH and VL chains of 3H1 and deduced the amino acid

sequences of the encoded proteins (24). For the T cells induced by 3H1 to recognize CEA-positive tumor cells, it is necessary for the amino acid sequences of 3H1 to have linear homology to CEA. To identify any cross-reactive peptides of 3H1 and CEA, we compared the amino acid sequences of 3H1 with those of CEA and found several regions of homology in the 3H1 heavy- and light-chain variable domains, as well as in the framework regions. To search for potential cross-reactive T-cell epitopes, a number of peptides based on 3H1/CEA homology were synthesized and were used as stimulants in cell proliferation assays, using PBMCs from the group of 3H1-immunized CEA-positive cancer patients in the adjuvant setting. Two partially homologous peptides, designated LCD-2 (from 3H1) and CEA-B (from CEA), were identified that generated strong proliferation responses in 10 out of 21 patients and were extensively studied in 5 of these individuals for over 1–2 yr. Analysis of the subtypes of the responding T cells demonstrated that primarily CD4+ T cells were stimulated by both 3H1 and these peptides (24).

Cytokines secreted by the stimulated T cells were either interleukin-2 (IL-2) or interferon- γ (24,29,67,69), suggesting that these are Th1 type of helper cells. Cytokine secreted by the Th1 cells invoked by anti-Id therapy may provide help to the cytotoxic T cells or to the natural killer (NK) cells.

6.3. Cytotoxic T Cells and NK-Mediated Immunity

In the L1210/GZL murine model, Raychaudhuri et al. (71) demonstrated the induction of tumor-specific cytotoxic T lymphocytes (CTLs) following immunization with the anti-Id antibody 2F10 mimicking an epitope of the mouse mammary tumor virus-encoded envelop glycoprotein gp52. The frequency of L1210/GZL tumor cell-reactive CTLs was similar in mice immunized with the anti-Id antibody or irradiated tumor cells when examined at the precursor level. Rather than using a classic Ab2 approach, Ruiz et al. (72) induced anti-Id network by immunization of mice with a monoclonal antibody (Pab-240) specific for mutated p53. Immunized mice produced IgG antibodies to p53 and mounted cytotoxic reaction to tumor cells bearing mutated p53. The idiotypically immunized mice were resistant to challenge with the tumor cells.

In cancer patients, evidence that anti-Id antibody can stimulate a CTL response was provided by immunization of melanoma patients with two different anti-Id antibodies mimicking high-molecular-weight proteoglycan antigen (73). Human leukocyte antigen (HLA)-A2-specific CTL responses were observed in 43% of the patients who appeared to express the HLA-A2 allele. Anti-Id antibody ACA 125, which mimics the ovarian tumor-associated antigen CA 125, also induced cellular immune responses that resulted in lysis of antigen-positive ovarian tumor cells (43). Although CTL assays in these two studies were performed with HLA-matched tumor cells, autologous tumor cells were not available and could not be used. Thus, other mechanisms of cytotoxicity could be involved in the lysis of the tumor cells. Durrant et al. (74) treated six patients with rectal cancer with the anti-Id antibody, 105AD7. PBMCs or lymph node cells from three of these patients caused significant killing of autologous tumor cells, which were shown not to be due to NK activity. The activities were only seen with immune cells from patients after immunization with 105AD7 and not before therapy. Enhanced NK activity was seen in three of six patients after the anti-Id therapy.

7. CONCLUSION AND FUTURE DIRECTIONS OF ANTI-ID CANCER VACCINES

Anti-Id vaccines represent an elegant approach to active specific immunotherapy. Anti-Id vaccines are very effective at stimulating both humoral and cellular antitumor immune responses, which are long-lasting. In this review we have discussed results where Ab2 were used as immunogens. Studies where the network cascade was activated by Ab1 administration were not included. Ab2 population induced by Ab1 administration is polyclonal and only a fraction of the Ab2 is likely to be Ab2 β type. Since not all Ab2 molecules are tumor protective (75), this approach of ASI may not be always effective.

Numerous clinical trials in colorectal carcinoma, melanoma, breast, and ovarian cancer patients have shown that anti-Id antibodies are nontoxic (25). In most cases anti-Id vaccines were effective in eliciting immune responses despite the absence of a strong adjuvant. Aluminum hydroxide precipitation, although considered weakly immunogenic, appeared to be quite adequate in eliciting immune responses. Aggregation of soluble idiotypic determinants by aluminum hydroxide precipitation are likely to help increase its antigenicity. In our experience, conjugation of anti-Id to KLH in combination with a strong adjuvant was necessary to raise optimal immunity in mice. As we moved to higher species, such as rabbits, we did not require KLH coupling and only a strong adjuvant was needed. In monkeys and humans we could use anti-Id vaccines after precipitation with the aluminum hydroxide.

Anti-Id antibodies are mostly murine in origin and by virtue of their xenogeneic origin, they are likely to elicit antibody responses in humans (human antimurine antibody [HAMA]). It was felt that HAMA generated may neutralize the mouse antibodies and diminish their half-life through rapid clearance (76). Although this may be true for passive intravenously administered murine immunoglobulins, it does not appear to be a problem with active immunotherapy. Increasing titers of Ab3 and Ab1' antibodies were observed after repeated therapy with mouse anti-Id antibodies in numerous clinical studies. It is likely that HAMA induced by therapy form complexes with the anti-Id antibodies, which are endocytosed by antigen-presenting cells and invoke antitumor responses (77).

An anti-Id antibody should be carefully chosen for use in clinical trial. Raychaudhuri et al. (75) generated six different anti-Id antibodies mimicking the mouse mammary tumor virus TAA antigen, gp52. All these antibodies serologically met the criteria of internal-image antigens. However, only one, 2F10, induced protective immunity and was effective in immunotherapy (75). Comparison of the amino acid sequences of these anti-Id antibodies showed that the sequences of the variable domains of the heavy chains were mostly homologous, but the sequences of the light chains varied considerably. CDR2 of the light chain of 2F10 showed considerable linear homology to a part of the nominal antigen, gp52. Thus, structural identity between an epitope of the antigen and a segment of the variable region of the anti-Id antibody (structural criterion) may predict the antitumor efficacy of an anti-Id antibody mimicking a protein antigen. For nonprotein antigens, the antitumor immunity most likely is antibody mediated.

Future studies should be directed at improving the antitumor activities of anti-Id antibodies. This can be done by the use of a cocktail of Ab2 antibodies, rather than a single one. For example, a large number of Ab2 antibodies directed against CEA have been generated (Table 1). The effect of a mixture of some of these can be compared against a

single Ab2. The heterogenous expression of a TAA in a tumor specimen poses a problem for the development of specific immunotherapy. As a result of therapy, immune escape variants are generated, dispelling any clinical benefit. Generation of these variants is less likely if the therapy is directed against multiple TAAs. A number of Ab2 antibodies exist for colorectal carcinoma mimicking a variety of TAAs (Table 1). Combination of these antibodies may prove to be more beneficial for immunotherapy compared to a single anti-Id antibody. The characteristics of the TAA are also important for the preparation of an anti-Id antibody for ASI. Anti-Id antibodies directed against growth factor receptors, such as Her2/neu, may be more effective than some other TAAs. We are characterizing several anti-Id antibodies directed against Her2/neu for exploring this possibility (46).

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13

Pox Viral Vaccines

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and Jeffrey Schlom, PhD*

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1. INTRODUCTION

Tumor-associated antigens (TAAs) are by definition either weakly immunogenic or functionally nonimmunogenic. Vaccine strategies must be developed in which the presentation of these TAAs to the immune system results in far greater activation of T cells than is being achieved naturally in the host. One approach to create an inflammatory milieu designed to trigger a robust immune response to TAAs involves the use of recombinant viral vectors to deliver the appropriate genetic material. Though several viral vector platforms are currently being evaluated, the pox virus family of vectors possesses several properties that make these vectors extremely attractive for use in anticancer vaccines. These include: a) the capacity to integrate the entire tumor antigen gene, parts of that gene, and/or multiple genes (including genes for costimulatory molecules and cytokines), b) the ability to infect antigen-presenting cells (APCs) allowing them to process the antigens, and c) the relative low cost. The ability to incorporate multiple transgenes within one vector is unique to these large virions, and allows for potential synergy of the incorporated gene products. This chapter will provide some insight into the strengths and weaknesses of pox viral vaccines, as well as the preclinical studies and early clinical trials of pox viral vaccine strategies for cancer therapy.

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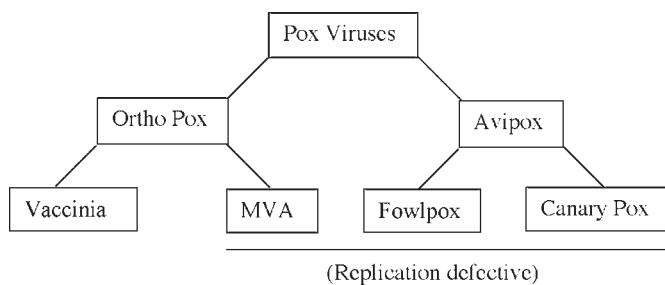


Fig. 1. The pox virus family contains two groups that have been studied as vectors for cancer vaccines. The orthopox group contains vaccinia, which replicates in mammalian tissue. MVA (modified vaccinia Ankara) is derived from vaccinia; however, it is missing the genetic material required for replication. The avipox vectors are replication defective in mammalian tissue.

Table 1
Properties of Pox Viruses

-
- Can insert a large amount of foreign DNA
 - Multiple transgenes (up to seven)
 - Will infect a large range of cells
 - Recombinant genes remain stably expressed
 - Replication accurate
 - Efficient post-translational processing
 - Recombinant proteins are highly immunogenic
-

2. PROPERTIES OF POX VIRUSES

Members of the Poxviridae family can be divided into those that are capable of replicating in mammalian species (e.g., *Orthopoxvirus* group) and those that can infect mammalian cells but cannot complete the replication process (e.g., *Avipoxvirus* group; see Fig. 1). There are advantages and disadvantages to the use of each as vectors. Some of the viruses that are replication competent have been extensively studied and have well-defined safety profiles (1). These vectors can continue to infect additional cells producing more TAA until they are eradicated by the immune system. Often vaccines associated with these replication-competent vectors are more immunogenic than those using replication-defective vectors. The replication-defective vectors, however, are theoretically safer because they can infect mammalian cells only once. Several properties of pox viruses have led to their extensive use as expression vectors, including their capacity for carrying large amounts of transgene DNA and their wide range of hosts (2,3). These viruses have several advantages, including the ability to make stable recombinant vectors with accurate replication and efficient post-translational processing of the transgene, and the fact that they do not integrate into eukaryotic DNA (see Table 1).

Pox viral vectors are unique in that their viral DNA replication and RNA transcription take place exclusively in the cytoplasm and can be demonstrated in enucleate cells. Because the vector's RNA is transcribed in the cytoplasm, the expression of any inserted transgenes requires pox virus promoters. Ideally, an open reading frame of the TAA is

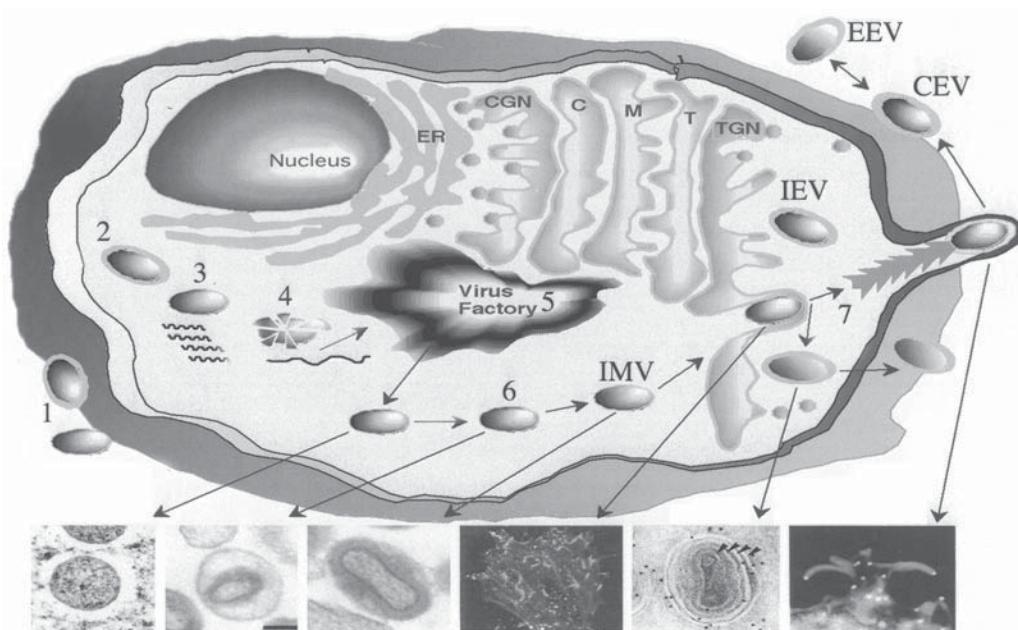


Fig. 2. A diagram of a vaccinia-infected cell is shown with an exaggerated view of the endoplasmic reticulum (ER), *cis*, *medial*, *trans* Golgi, and the *trans*-Golgi network (C, M, T, and TGN, respectively). The major stages of the virus life cycle are listed. Following late gene expression, previrion forms assemble to form intracellular mature virus (IMV). The IMV is targeted to the TGN and following envelopment, intracellular enveloped virus (IEV) is formed. IEV are propelled to the cell surface by the polymerization of actin filaments. Once released, the virus may remain attached to the membrane as cell-associated enveloped virus (CEV) or be released into the medium as extracellular enveloped virus (EEV). Reproduced with permission from reference 10.

juxtaposed to a viral promoter resulting in gene expression levels typical for that promoter. This cytoplasmic replication abolishes many safety concerns inherent in other recombinant viral vaccines that replicate in the nuclear compartment in close proximity to cellular genes.

When infecting a cell, a pox virus first undergoes endocytosis and the viral core is released into the cytoplasm (see Fig. 2). The viral transcriptase is then activated and viral mRNA is produced inside the cytoplasm within minutes after infection. This process also shuts down normal cellular transcription inside the nucleus. Among the first genes transcribed are ones encoding for proteins that complete the uncoating of the viral core and other “early” genes that are transcribed prior to reproduction of viral DNA. There are about 100 of these early genes, many with unique promoters, that can be replaced with transgenes encoding TAAs. With vaccinia infection in humans, viral DNA replication occurs within 1.5–6 h following infection. The process of virion assembly and mature particle budding from the membrane completes the life cycle. Multiple virions budding simultaneously from the membrane causes cellular lysis.

With pox vectors that are replication defective in mammalian species (e.g., fowlpox), the early genes are transcribed; however, viral DNA cannot be replicated nor can the late viral gene products that aid in assembly be made. Thus transgenes on early promoters are made within 30 min after infection with peak expression of proteins occurring in about

Table 2
**Serious Complications of Vaccinia in Patients at Least 20 Yr of Age on Revaccination
in a 10-State Survey (1968)^{a,b}**

auto-inoculation	25.0/10 ⁶ vaccinations
generalized vaccinia	9.1/10 ⁶ vaccinations
eczema vaccinatum	4.5/10 ⁶ vaccinations
post-vaccinia encephalomyelitis	4.5/10 ⁶ vaccinations
vaccinia gangrenosum	6.8/10 ⁶ vaccinations

Note. It should be noted that these patients were given the vaccine by scarification without occlusive dressings and that patients with immunodeficiency states or skin conditions were not excluded, as is done in current clinical trials.^{b,c}

^a Lane JM, Ruben FL, Neff JM, Millar JD. Complications of smallpox vaccination, 1968: results of ten statewide surveys. *J Infect Dis* 1970; 122:303–9.

^b Vaccinia (Smallpox) Vaccine Recommendations of the Immunization Practices Advisory Committee (ACIP) *MMWR* 40(RR14): 1–10. 12/13/1991

<http://www.cdc.gov/mmwr/preview/mmwrhtml/00042032.htm>

^c Vaccinia (Smallpox) Vaccine Recommendations of the Advisory Committee on Immunization Practices (ACIP), 2001 *MMWR* 50(RR10): 1–25 06/22/2001

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<http://www.cdc.gov/mmwr/PDF/RR/RR5010.pdf>

6 h. During this process few viral proteins are translated, decreasing the potential for a host immune response to the vector. Cells infected with these virions also shut down cellular transcriptions and the cells slowly die over 14–21 d, generally by apoptosis. These cells do not release infectious virions.

2.1. Replication-Competent Pox Viral Vectors

One of the most thoroughly investigated pox viral vectors is vaccinia. This virus has been used since 1796, when Jenner first demonstrated the ability of vaccinia to protect against subsequent variola inoculation and thus protect against smallpox. This technique was widely applied with over a billion doses given worldwide, thereby leading to the eradication of smallpox (4). The intensity of this vaccination program has led to the compilation of a highly accurate safety profile of vaccinia (*see Table 2*). These vectors can rarely cause potentially life-threatening conditions in severely immunocompromised individuals.

Vaccinia virus is the prototype pox virus and possesses a complex dsDNA genome, containing 186 kb encoding over 200 proteins enclosed in a bilipid envelope. Many of the viral genes can be deleted and replaced by TAA gene(s), except for the inverted terminal repeat sequences within the genome that are responsible for viral replication.

Recombinant vectors are achieved by homologous recombination after transfection of vaccinia virus-infected cells with plasmid DNA constructs (5). This virus is replication competent, and cytopathic, with a broad host range and the capability to infect both vertebrate and invertebrate hosts (6). The advantages of using vaccinia viruses for gene transfer include their ability to accommodate inserts of at least 25 kb, allowing for large or multiple (up to seven) gene inserts; their ability to infect cells regardless of their mitotic state; and their cytoplasmic replication, which avoids any potential for chromosomal mutation on insertion (7,8). Additionally, mammalian proteins expressed from transgenes inserted in vaccinia vectors are processed and modified in a manner comparable to that

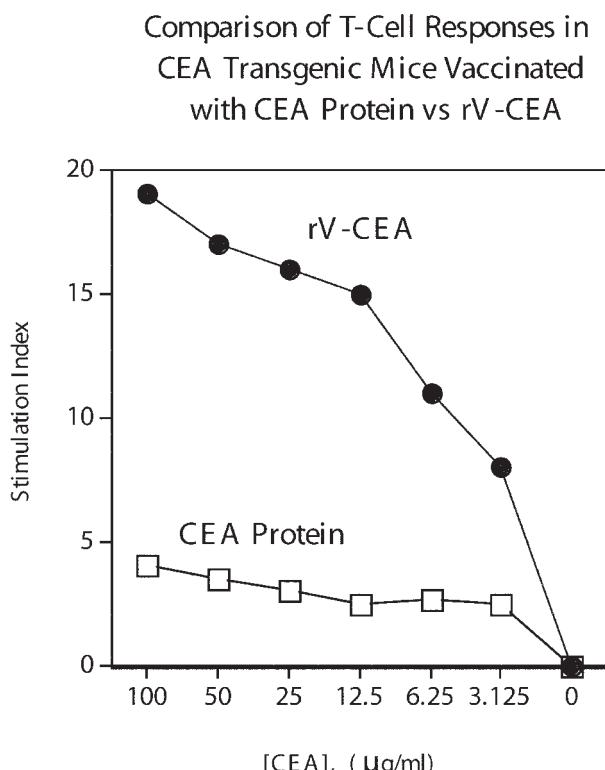


Fig. 3. Carcinoembryonic antigen (CEA)-specific splenic CD4+ proliferative responses from CEA Transgenic mice immunized with rV-CEA (2x) or CEA protein. CEA Tg mice (H-2D) were administered 10^7 pfu rV-CEA 2x (closed triangles) by tail scarification. Another group of CEA Tg mice was administered 100 μg CEA in 100 μL adjuvant (Superdetox) (2x) by tail scarification (solid circles). Fourteen days later, mice were scarified, splenic T cells were isolated, and the proliferative responses to soluble CEA were measured by a T-cell lymphoproliferative assay. From references 13 and 79 with permission.

when expressed in their native environment (9,10). These foreign antigens are presented to the immune system with the large number of proteins produced by the vector itself, which likely is responsible for the significant inflammatory response to the pox virus vector. In turn, this inflammatory process can lead to an environment of cytokine production and T-cell proliferation, which may act to further amplify the immune response to the foreign antigen. This process favors induction of a cell-mediated immune response and humoral responses to the foreign antigen.

However, vaccinia has been widely used as a vaccine for smallpox; therefore, administration of vaccinia virus for gene transfer could be impaired by a robust host antivaccinia immune response. In contrast, this potential disadvantage (intrinsic antigenicity) can be exploited to elicit an immune response against TAAs to activate an antitumor immunotherapeutic response *in vivo* (11). In fact, one of the main advantages of using recombinant vaccinia viruses to develop cancer vaccines is that when a gene for a weakly immunogenic protein is inserted into recombinant vaccinia and used as an immunogen, the recombinant protein is much more immunogenic as a vaccine than the use of that protein with adjuvant (12–14). A striking example of this is seen in Fig. 3, which shows

Table 3
Selected Clinical Trials

Vaccine	Patients	Phase	Immune Response	Clinical Response	Reference
rV-CEA	26	I	Y	N	18
rV-PSA	33	I	Y	N	22
MVA-MCU-1/IL-2	9	I	Y	Y ^a	32
rV-CEA → avi-CEA	18	I	Y	Y	37,45
Avi-CEA/B7.1	60	I	Y	N	61
rV-PSA + rV-B7.1 → rF-PSA	27	II	Y	Y ^b	25
rV-CEA(6D)/TRICOM → avi-CEA(6D)/TRICOM	32	I	Y	Y ^c	69

^aOne patient had a decrease in CEA with stable disease for 10 wk.

^bOne patient with a sustained PSA decrease from 8.7 ng/dL to 0.2 ng/dL with vaccine alone.

^cOne patient with pathologic complete response.

that two injections of carcinoembryonic antigen (CEA) protein in adjuvant generated little, if any, of an immune response to CEA in a CEA transgenic mouse. CEA is a 180 kD oncofetal glycoprotein that is expressed in normal colonic mucosa and overexpressed in virtually all colorectal adenocarcinomas and most adenocarcinomas of the pancreas, stomach, breast, and lung (15–17). This would be expected because the host is seeing CEA as a “self” antigen. However, when the recombinant vaccinia virus containing the CEA transgene (designated rV-CEA) is administered one or two times, a strong CEA-specific T-cell response is elicited (14). The likely reason for this is that a strong inflammatory response is generated by the host against vaccinia proteins, which leads to an environment of cytokine production and T-cell proliferation.

In a trial with 26 patients who had advanced colon carcinoma, recombinant vaccinia CEA (rV-CEA) vaccine given monthly for 3 mo was well tolerated (see Table 3) (18). Though no clinical responses were observed, this was the first demonstrated generation of a human cytolytic T-cell response to specific epitopes of CEA. These studies also showed that when the rV-recombinant was administered at 10⁸ pfu to patients with prior smallpox vaccines, all patients demonstrated a “take,” i.e., erythema, to the vaccine and patients mounted an immune response to the transgene (CEA). Other trials with rV-CEA also showed lack of toxicity (19), antibody responses (20), and apparent equivalence of subcutaneous and intradermal injections (21).

Prostate-specific antigen (PSA) is a 130 kD serine kinase that is expressed virtually only in prostate cells. Early trials with recombinant vaccinia PSA showed that it was well tolerated and could elicit specific cellular immunity (22–24). These trials added to evidence from preclinical models that further increases in antigen-specific immune responses were limited after the second and third vaccination, presumably due to the vigorous immune response to the vaccinia proteins.

Avipox vectors, which do not express the late viral antigens in mammalian cells, and thus are replication defective in humans, do not have a significant immune response generated to their viral proteins. Thus, trials using heterologous prime and boost strategies were developed with recombinant vaccinia vectors and recombinant avipox vectors.

An ongoing randomized phase II clinical trial has demonstrated that rV-PSA admixed with rV-B7.1 followed by monthly rF-PSA can generate PSA-specific T-cell responses and, in one patient, a sustained PSA decline from 8.7 ng/dL on study to 0.19 ng/dL after four vaccinations. This vaccine is given with granulocyte-macrophage colony-stimulating factor (GM-CSF) at the vaccine site and low-dose interleukin-2 (IL-2) sc 1 wk later at a distant site (25).

2.2. *Replication-Defective Pox Viral Vectors*

The avipox viruses represent potentially attractive vectors for use in cancer vaccines. Though the immunogenicity of the inserted transgene may not be as potent as a transgene in a vaccinia virus, avipox viruses such as fowlpox and ALVAC can be administered numerous times to enhance immunogenicity (26–28). Since they are replication defective, induction of any host immune responses should be relatively inconsequential. Avipox viruses are also distinguished from vaccinia in that the inserted transgene is expressed in infected cells for up to 3 wk before cell death ensues. In a vaccinia-infected cell, the transgene is expressed for 1–2 d until cell lysis, and for approximately 1 wk in the host until virus replication is arrested by host-immune responses.

Modified vaccinia Ankara (MVA) is a replication-defective pox virus derived from vaccinia following 500 passages in chicken embryo cells. It has been employed in many experimental studies and has now been administered to more than 120,000 humans without apparent side effects (29). Recently, this virus has been molecularly characterized and has been found to have lost several genes involved in host-range determination and possible immune system suppression. Though MVA efficiently infects human cells and expresses both early and late genes, it is incapable of producing infectious progeny in mammalian cells. MVA recombinant viruses have been shown to be highly immunogenic in both rodents and primates. An MVA vector expressing the pan adenocarcinoma antigen MUC-1 and IL-2 genes has been tested in several preclinical studies with infection of human dendritic cells (DCs). These studies have shown that MUC-1 and IL-2 encoded by MVA can be expressed and IL-2 is functional (30). Preliminary results of a similar dual-gene construct in vaccinia have suggested that the vector was well tolerated and produced immunologic responses in prostate and breast cancer patients (31,32). Together, these and other studies lay the basis for clinical trials with MVA vectors.

3. PRIME AND BOOST WITH POX VIRUSES

A number of investigators have now demonstrated the advantage of priming with vaccinia recombinants and boosting with immunogens such as recombinant protein, peptide, or recombinant fowlpox or avipox vectors to enhance immune responses in cancer vaccine models (27,33–35). The experimental studies that involved priming with recombinant vaccinia and boosting with recombinant avipox also demonstrated that the host immune response to the transgene increased with continued booster vaccinations (27).

It has now been demonstrated through both preclinical studies (27,33,36) and recent clinical studies (37,38) that optimal generation of specific T-cell responses can be achieved employing a heterologous prime-and-boost strategy. This has been demonstrated using a primary vaccination with a recombinant vaccinia virus expressing human CEA (designated rV-CEA) and a boost or multiple booster vaccinations with recombinant

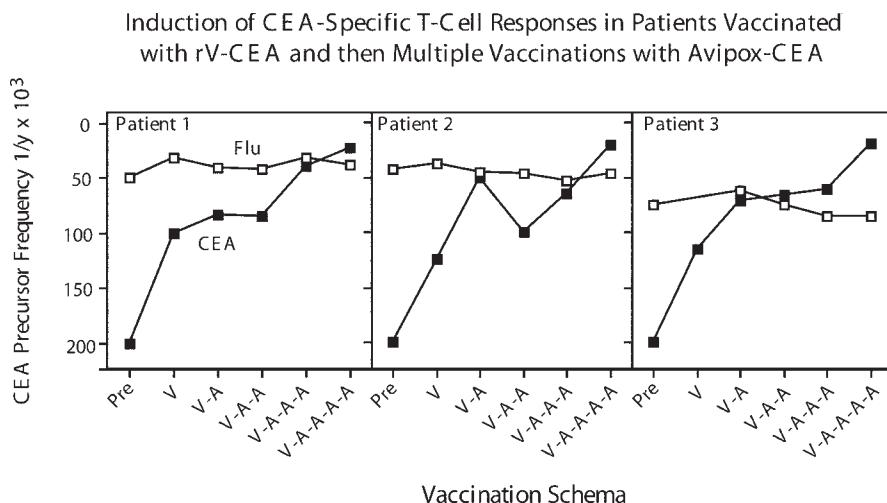


Fig. 4. Induction of CEA-specific CD8+ T-cell responses in patients following CEA-based vaccination. Patients with advanced carcinoma were vaccinated with rV-CEA(V) followed by one to four monthly booster vaccinations with avipox-CEA(A) recombinants. Peripheral blood mononuclear cells (PBMC) were obtained prior to vaccination and 1 mo after each vaccination. An overnight ELISPOT assay (to eliminate artifacts of in vitro stimulation) was used to measure response to a 9-mer CEA peptide (closed squares) and the control 9-mer Flu peptide (open squares). From ref. 37.

avipox virus expressing CEA (avipox-CEA). In these studies (36,39), mice were first immunized with wild-type vaccinia virus (to simulate patients who had previously received vaccinia as a smallpox vaccine). As long as an adequate dose of rV-CEA was administered, an immune response could be achieved to CEA. When these mice received a second vaccination of rV-CEA, only a small increase in CEA-specific T-cell responses could be achieved. When avipox-CEA was administered as a booster vaccination, however, a marked increase in CEA-specific T-cell responses was achieved; this result was far superior to that achieved when multiple injections of either rV-CEA or avipox-CEA were given. It has now been shown in murine models and in patients that recombinant avipox viruses can be given multiple times with subsequent increases in immune response to the TAA transgene (see Fig. 4; 36,37,40). This is a result of the fact that the transgene is expressed via an early pox virus promoter (41), whereas many structural proteins of the avipox virus that are normally downstream from late pox virus promoters are not expressed in mammalian cells. Thus, even in the face of some anti-avipox immune responses, no neutralization of the avipox virus even after multiple vaccinations is evident (40). Furthermore, when mice were administered rV-CEA as a prime and recombinant CEA protein as a boost, T-cell responses to CEA were greater than those achieved when either immunogen was used alone (33). This concept of heterologous prime-and-boost immunization is now also being validated in other model systems employing vaccines for infectious agents and cancer (28,42–44).

In an effort to determine which heterologous prime-and-boost regimen to use in humans, a small randomized trial was conducted to compare giving the rV-CEA as the initial priming vaccination followed by boosting with avipox-CEA (VAAA) with giving

the three vaccinations with avipox-CEA first followed by rV-CEA (AAAV) (37). This study showed that the immune responses seen in the VAAA arm were much better than those in the AAAV arm. Furthermore, continued follow-up of these patients revealed that although there were only nine patients in each arm, at the time of a recent presentation five of nine patients were alive on the VAAA arm (2-yr survival estimate $67 \pm 19\%$); in contrast, in the AAAV arm, zero of nine patients were alive (2-yr survival estimate $0 \pm 0\%$) (45). This survival difference was related to the immune response with those patients who had at least a 2.5-fold increase in their CEA-specific T cells living longer ($p = 0.03$).

4. T-CELL COSTIMULATORY MOLECULES

The induction of a vigorous T-cell immune response requires at least two signals. Signal 1 is mediated through a peptide major histocompatibility complex (MHC) on the surface of the APC that interacts with the T-cell receptor; signal 2 is mediated through the interaction of a T-cell costimulatory molecule(s) on the surface of the APC with its ligand on the T-cell surface. In order to provide additional costimulatory molecules for the APCs, the genes for costimulatory molecules can be added into pox vectors. The need for T-cell costimulation is especially important in the presence of a weak signal 1, such as in the case of a TAA. To date, numerous T-cell costimulatory molecules have been identified (46–55).

Preclinical studies have revealed significantly improved specific T-cell activation and tumor killing in murine treatment models with the addition of costimulatory molecules encoded by pox vectors (36). Most of these studies have been conducted in murine models, and the vast majority of the studies have been conducted with the T-cell costimulatory molecules B7-1 and B7-2, using either retroviral vector constructs or antibodies directed against the T-cell ligand CTLA-4. Many T-cell costimulatory molecules have now been inserted individually and in combinations into pox virus vectors. Initial preclinical studies involved the use of the weakly or nonimmunogenic murine carcinoma tumor MC38. When this tumor is x-irradiated and used as an immunogen, subsequent challenge with live MC38 tumor cells results in rapid tumor growth and death of animals (56). These studies demonstrated that when MC38 cells are infected with recombinant vaccinia viruses expressing either B7-1 or B7-2 costimulatory molecules, then x-irradiated, and used as immunogens, protection against tumor challenge is achieved (57); the administration of anti-CTLA-4 antibody had no effect on inhibition of this tumor (56). Using the same model, a study was set up to compare the relative efficacy of recombinant vaccinia vs retroviral vectors. Recombinant retrovirus expressing B7 (R-B7-1) and rV-B7-1 were used to infect MC38 carcinoma cells. When irradiated tumor cell vaccines were given to mice bearing experimental MC38 lung metastases, the MC38/rV-B7 vaccine was shown (56) to be significantly more effective in the treatment of pulmonary metastases than the MC38/R-B7-1 vaccine ($p = 0.0014$). This was true in mice that were either vaccinia naïve or vaccinia immune.

4.1. Admixing vs Dual-Gene Constructs of Recombinant Pox Vectors

One important consideration in the use of vectors to deliver both signal 1 and signal 2 to the APC is that both must be expressed on the same cell for effective T-cell activation. Recombinant avipox viruses expressing both signal 1 and one or more costimulatory molecules have been constructed. Because vaccinia is a replication-competent virus,

studies were conducted to determine whether admixtures of various recombinant vaccinia viruses could be used to efficiently and rapidly determine the effects of different T-cell costimulatory molecules. Studies have shown (58) that an admixture of rV-CEA and rV-B7 is just as effective as the dual-gene construct rV-CEA/B7 in (a) infecting APC in vitro and (b) generating CEA-specific T-cell responses in vivo. To accomplish this, both recombinant viruses were required to be administered at doses of 5×10^7 pfu or greater and administered sc or id. This phenomenon of admixing was also used to demonstrate (59) a therapeutic antitumor response of experimental lung metastases expressing the MUC-1 breast cancer-associated gene. When tumor-bearing mice were primed with an admixture of rV-MUC-1 and rV-B7 followed by two boosts with rV-MUC-1, there was a significant reduction in pulmonary metastases ($p < 0.0001$); this correlated with 100% survival as compared with no significant antitumor effect when using multiple rV-MUC-1 vaccinations without the costimulatory molecule (59). Clinical studies using admixtures of rV-PSA and rV-B7.1 as a prime vaccination followed by boosting with rF-PSA are now in progress (25). Several clinical trials have shown that the addition of B7.1 into nonreplicating (avipox-CEA-B7.1) viruses has resulted in vaccines that produce little toxicity and generate specific immune responses (60,61).

4.2. Analysis of the Potency of Different T-Cell Costimulatory Molecules

Previous studies have demonstrated that B7-1 and B7-2 act in a similar fashion in enhancing T-cell responses and antitumor immunity (57). Studies were then conducted in which three other T-cell costimulatory molecules were evaluated for their ability to enhance antigen-specific T-cell responses and antitumor T-cell responses; ICAM-1, LFA-3, and CD70 were chosen for evaluation because each has an individual ligand on T cells. In a series of three recent publications, it was shown that rV-ICAM-1 (62), rV-CD70 (63), and rV-LFA-3 (64) each had the ability to enhance T-cell responses and/or antitumor activity. These studies were conducted either by using recombinant vector-infected x-irradiated tumor cells as vaccine or by admixing with rV-CEA as a vaccine.

4.3. TRICOM Vectors

The use of pox virus vectors has provided a unique opportunity to evaluate the possibility that multiple costimulatory molecules can have either additive or synergistic effects in further activating T cells. This is because the different costimulatory molecules must all be expressed on the same cell as the TAA for the phenomenon to be effective, and recombinant pox viruses can be constructed in which each transgene could be driven by a different pox virus promoter. B7-1, ICAM-1, and LFA-3 T-cell costimulatory molecules were chosen to comprise this triad of costimulatory molecules (designated TRICOM) because they have all been previously shown to bind different ligands on the T cell and signal through different pathways (65–67).

Recombinant vaccinia and avipox vectors have been designed, constructed, and generated, containing one, two, or the three transgenes for B7-1, ICAM-1, and LFA-3 that are driven by different pox virus promoters (68). The MC38 murine carcinoma cell line was employed as an APC in initial studies because it lacks expression of any known costimulatory molecules. MC38 cells infected with TRICOM vectors were shown to express all three costimulatory molecules on their surface within 5 h. Moreover, the levels of expression of all three costimulatory molecules on cells, as determined by mean

fluorescence intensity, were similar whether using TRICOM vectors or vectors expressing individual costimulatory molecules. Studies were first conducted (68) to determine the level of activation of naïve T cells in vitro using concanavalin A (ConA) as a pharmacological signal 1. A greater than 10-fold increase in T-cell proliferation was observed using cells infected with either rV- or avipox-TRICOM vectors as opposed to cells infected with vectors expressing any single costimulatory molecule.

To determine the effects of costimulation by single or multiple costimulatory molecules on cytokine production, purified CD4 and CD8 T cells were cocultured with various stimulator cells expressing B7-1, ICAM-1, or LFA-3, or expressing TRICOM. Marked increases in the production of IL-2 by CD4 cells, and interferon- γ (IFN- γ) and IL-2 by CD8 cells, were observed with the TRICOM vectors (68). Little, if any, increase in IL-4 production was observed. Thus the TRICOM vectors appeared to preferentially stimulate production of Th1 cytokines.

The pronounced levels of T-cell activation seen with the use of TRICOM vectors suggests the possibility that overstimulated T cells would undergo program cell death, i.e., apoptosis. T cells were thus activated with Con A for signal 1 and cultured with MC38 cells that had been infected with V-WT, rV-B7, or rV-TRICOM. T cells activated with either MC38/B7 or MC38/TRICOM actually exhibited less apoptosis than T cells activated with either MC38 or MC38/V-WT (68). There are several ongoing clinical trials looking at CEA-TRICOM recombinant pox vectors (69).

5. DENDRITIC CELL VACCINES WITH POX VECTORS

The role of the DC in the induction of antigen-specific T-cell responses has focused the attention of many investigators on the potential efficacy of these cells in the immunotherapy of cancer and infectious agents. The use of DCs, however, has been limited by their trace levels in peripheral organs (70). Rad et al. showed that by infecting bone marrow progenitor cells (BMPCs) or DCs with vectors containing TRICOM (either avipox or vaccinia) there was significant enhancement of both the naïve and effector CD4+ and CD8+ T-cell populations in mice (71). Thus DCs or BMPCs can be made more potent APCs by the TRICOM-vector driven overexpression of three costimulatory molecules. This has direct implications for the use of such technology with human cells using TRICOM vectors containing human B7-1, ICAM-1, and LFA-3 as transgenes. Currently, human DCs for human clinical trials are being prepared from CD34+ progenitor cells mobilized from the bone marrow via treatment with Flt-3 ligand (72). TRICOM vector-infected CD34+ progenitor cells could potentially be made more potent APCs to the level approaching DCs.

6. CYTOKINE EXPRESSION DRIVEN BY POX VECTORS

Both orthopox (vaccinia) and avipox (fowlpox and canarypox) vectors have been used for the expression of GM-CSF. Previous studies have shown that direct injection of rV-GM-CSF into tumor lesions will enhance antitumor effects both in experimental animals (73) and in clinical studies in melanoma patients (74). On the other hand, fowlpox expresses transgenes for 14–21 d. A recent study in mice has compared the biological activity of recombinant GM-CSF with that of fowlpox expressing GM-CSF (40). A single injection of rF-GM-CSF led to significantly higher numbers of APCs, particularly DCs, in regional lymph nodes when compared with four daily injections of GM-CSF. In

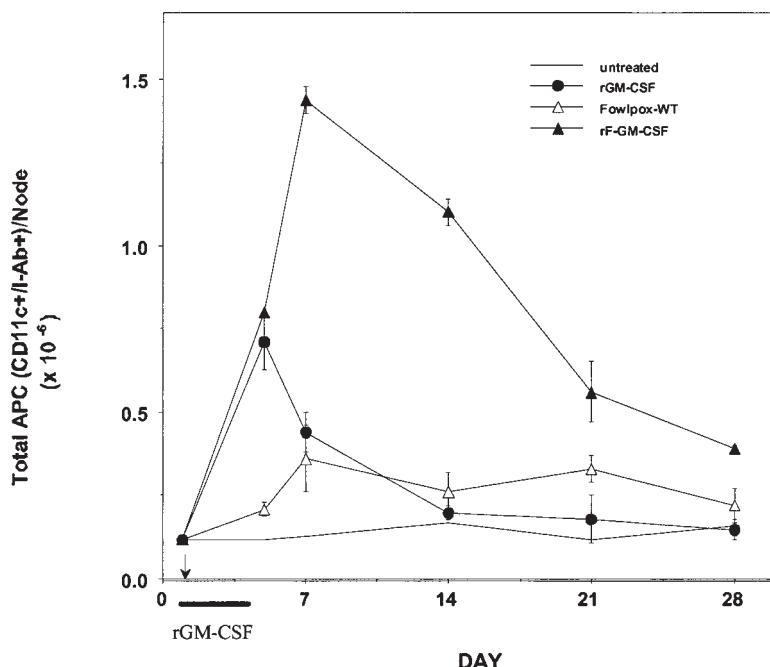


Fig. 5. Total number of APCs per lymph node in mice treated with fowlpox-GM-CSF or rGM-CSF. B6 mice (8–12/group) were administered 10^8 pfu (arrow) of rF-GM-CSF or fowlpox-WT. Recombinant GM-CSF (20 µg) was given to 15 mice for four consecutive days and control mice were given HBSS. Lymph nodes were removed and the total number of CD11c⁺/I-Ab⁺ cells were determined by flow cytometry. From ref. 40 with permission.

addition, although no increased expression of APCs could be found in regional lymph nodes 9 d after the last of the four daily injections, increases in APC expression in regional lymph nodes were still observed 28 d after the administration of rF-GM-CSF (*see* Fig. 5). Equally significant, Kass et al. showed that rF-GM-CSF may be given repeatedly with this same level of biologic effect despite the formation of anti-fowlpox antibodies (40). This provides further evidence that antivector responses against avipox viruses are not neutralizing. No anti-GM-CSF antibodies were detected in these mice after three vaccinations.

7. FUTURE DEVELOPMENT OF RECOMBINANT POX VIRUS VACCINES

The future development of recombinant pox virus vaccines holds considerable promise for cancer management. Virtually all of the clinical studies described above have been carried out in patients with relatively advanced cancers. These patients most likely have subtle but depressed immune responses, which have been exhibited in two ways: (a) via downregulation of the ζ chain of the T-cell receptor and (b) in the cytokine profile of T cells obtained from advanced cancer patients (type 2 profile) compared with a type 1 profile from healthy individuals. Thus, the full potential of these vaccines has yet to be defined. The advantage of diversified prime and boost protocols employing pox virus recombinants has now been demonstrated preclinically and in the clinic. It is not known,

however, how many boosts or the timing interval between the boosts using replication-defective avipox viruses or MVA are optimal.

Modification of tumor-associated immunodominant epitopes such as those of gp100 and CEA have been shown to enhance immune responses in vitro (75) and in patients (76–78). The use of recombinant pox viruses containing these modifications in tumor-antigen transgenes may enhance immune responses in patients. The use of cytokine genes as transgenes in recombinant pox viruses either alone or with tumor-antigen genes is currently being explored, as is the administration of recombinant cytokines such as GM-CSF and IL-2 in vaccine protocols containing recombinant pox viruses.

Perhaps the most intriguing advantage of using recombinant pox virus vectors in anticancer vaccines is the ability to insert multiple transgenes; this can include the use of multiple tumor-antigen genes, cytokine genes, and/or T-cell costimulatory molecule genes. The observation that the vast majority of tumor-associated genes are weak immunogens supports this approach. Underscoring these vectors' significant potential as vaccines is the demonstration that recombinant pox viruses can efficiently express three T-cell costimulatory molecules in the same APC and activate T cells to levels not previously achievable with the use of any one or two of these molecules. Modes of application of pox vector-based vaccines potentially include administration to patients as a classical vaccine, direct injection into tumor masses, infection of whole tumor cells prior to irradiation as a vaccine, or the in vitro infection of APCs such as DCs as vaccines.

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CONTENTS

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1. ADENOVIRUS-BASED VECTORS

The fact that adenovirus (Ad) vectors have been utilized in over 164 clinical trials to date (at least 644 subjects) substantiates the claim that these vectors are potentially useful in a variety of gene transfer strategies (1). Ad vectors are constructed in a manner such that one can insert desired recombinant DNA sequences into the E1 region of the wild-type Ad genome (Fig. 1). Since the E1 genes only encompass a small fraction of the Ad genome, approx 90% of the wildtype Ad genome is retained in the resultant [E1-]Ad vector. For a complete review of the numerous methods to genetically engineer a first-generation, or [E1-]Ad-based vector, see Imperiale et al. (2). The recombinant [E1-]Ad vector genomes are introduced into human cells that express the E1 proteins *in trans*, allowing for helper virus independent [E1-]Ad vector replication and packaging, and high-level production (3).

[E1-]Ad vectors have a number of positive attributes; one of the most important is their “relative” ease for scaled up, good manufacturing practices (cGMP) production, due to helper virus-independent (HVI) growth. Additionally, since Ad vectors do not integrate (Ad vector genomes can express genes episomally, without the requirement for integration), the risk for integration and germ-line transmission is extremely low; this does however limit use of these vectors to non-rapidly dividing cell populations (4). Importantly, Ad vectors can transduce a variety of tissues *in vivo*, including nondividing, terminally differentiated cells such as those found in liver, kidney, muscle (skeletal and cardiac), respiratory, and nervous system tissues as well as cells *ex vivo*, including

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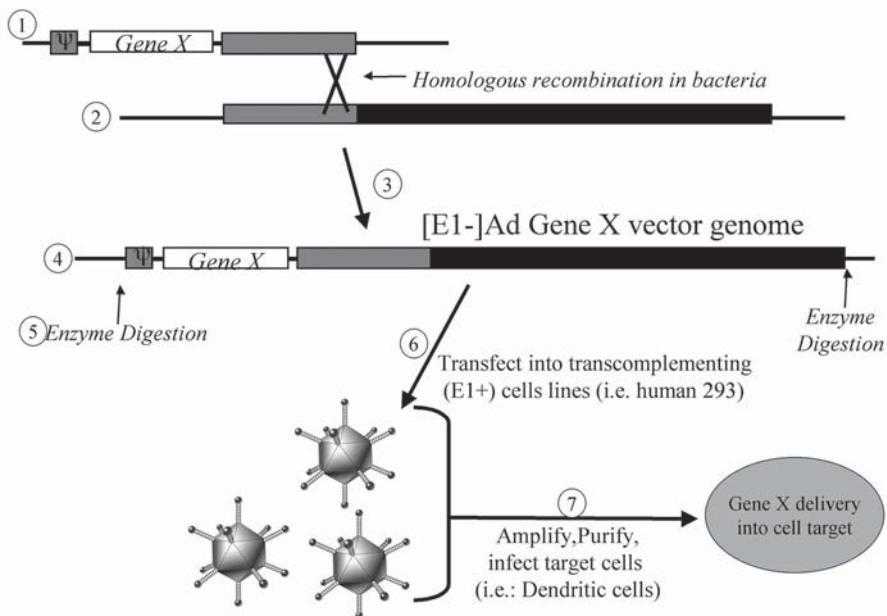


Fig. 1. Schematic representation of steps required to generate an [E1-]Ad vector. 1: shuttle plasmid containing gene of interest. Gene X represents a DNA sequence of interest. Note that the Gene X gene mini-gene cassette must include appropriate expressional control elements, including at least an appropriate enhancer/promoter element and polyadenylation signals \pm intronic signals \pm translational enhancer elements. Finally, note that the Gene X mini-gene cassette is flanked by Ad genomic sequences represented by \blacksquare . 2: Bacterial plasmid containing areas homologous to shuttle plasmid, as well as rest of Ad genome; latter sequences represented by \blacksquare . 3: Homologous recombination in recombinogenic bacteria results in generation of [E1-]Ad Gene X vector genome embedded in a bacterial plasmid. 4: [E1-]Ad Gene X vector genome embedded in a bacterial plasmid. 5: Restriction enzyme digestion of [E1-]Ad Gene X vector genome containing plasmid releases linear Ad genome from bacterial plasmid sequences. 6: Transfection of appropriately linearized [E1-]Ad Gene X genome into E1-expressing packaging cell line allows wild-type Ad life cycle to ensue, and packaging of recombinant Ad vector genome into Ad capsids. 7: Serial passaging of the Ad vector within increasing numbers of packaging cells allows for large-scale purification and downstream usage either ex vivo or in vivo. See He et al. for a specific method based upon the above schema (47).

dendritic cells. Conventional Ad vectors have a large carrying capacity, that theoretically approaches 7.5 kb with full deletion of the E1 and E3 genes (the E3 genes are nonessential for Ad growth in vitro). In this chapter, we will refer to [E1-,E3-]Ad vectors simply as [E1-]Ad.

2. ADENOVIRUS USE IN CANCER VACCINE STRATEGIES

With so much known about Ad biology, it was a natural step to use recombinant Ad for vaccination purposes. As a first example, [E1-]Ad vectors have been engineered that express genes encoding epitopes (or whole proteins) derived from a number of pathogens, including (but not limited to): malaria, bovine herpesvirus (type 1), foot-and-mouth

disease virus (FMDV), measles virus, and HIV (human immunodeficiency virus) (5–7). Vaccination with these Ad vectors has shown positive efficacy in preventing viral infection upon challenge with each of the respective viruses.

The potential benefits provided by the use of Ad vectors as vaccines for the prevention of viral illnesses coincide with simultaneous efforts to use Ad vectors to vaccinate against various types of cancer. The first use of Ad against cancer occurred shortly after its discovery in 1953. In this experiment, concentrated wild-type Ad were intravenously administered into subjects affected by cervical cancer. The hypothesis was that the lytic properties of Ad infection of cancer cells might slow or halt the cancer's progression, a popular notion of the period (8). Of the subjects treated, 65% reportedly had evidence of necrosis of cancerous tissue, without appreciable side effects. Autopsy findings demonstrated that the Ad had effects only on the cervical tumors and was not able to affect growth and metastases of pelvic tumors. Thus, although showing some promise, the lytic properties of Ad did not seem sufficient to effectively fight disseminated cancer.

A resurgence however has occurred in the use of Ad to treat cancer, as a result of both advancements in understanding of cancer biology combined with the ability to generate Ad vectors. For example, Ad vectors have been constructed that express a variety of immunostimulatory genes, tumor-suppressor genes, tumor repair genes, ribozymes, antisense oncogenes, apoptosis-inducing or toxic genes, and other genes thought to be critical in combating the growth of cancerous cells. Prodrug-sensitizing and tumor-specific, semireplicative Ad vectors have also been developed, representing other approaches to Ad-based cancer therapy. In these approaches, tumor nodules are typically directly injected with the respective vector, to achieve at least localized, if not systemic anticancer effects. For a more detailed discussion of these types of strategies, a number of reviews are recommended (9,10).

An alternative approach to using recombinant Ad vectors to combat cancer involves its use as a traditional vaccine, to immunize individuals against cancerous cells. This strategy was made possible by the discovery of various tumor-associated antigens (TAAs), which may provide immunological targets to a number of types of cancer via a traditional vaccine approach. One of the first demonstrations of this approach utilized an [E1-]Ad vector expressing the EBV gp340/220, in an attempt to induce protective immunity in cottontop tamarins challenged with Epstein-Barr virus (EBV)-induced lymphomas (11). In the study, three intramuscular injections of the recombinant Ad vector were sufficient to protect all immunized animals against subsequent tumor challenge. This tumor-specific protective effect was also seen when a recombinant Ad encoding beta-galactosidase was used in a vaccination of BALB/c mice (12). In this study, the ex vivo infection of splenocytes with an [E1-]Ad expressing the bacterial beta-galactosidase gene could induce a dramatic regression of established pulmonary metastases in the mice. A caveat of this study was that the tumor cells (CT26.CL25) were previously engineered to also express the bacterial β -gal-expressing gene. The bacterial beta galactosidase gene may have enhanced the repertoire of immune targets in this scenario, acting as another foreign epitope to the host animal's immune system. Other limitations to the approach were also noted; for example, mice bearing established tumors, subsequently vaccinated with the same [E1-]Ad vector, experienced no significant regression of tumor, even when simultaneously treated with interleukin-2 (IL-2). Though limited by the immuno-logic assays of the day, and use of tumor cells lines expressing potentially foreign transgenes, both of these early studies showed the potential of Ad as an effective cancer vaccine.

Another obvious cancer target can be found in cervical cancers induced by the human papilloma virus (HPV). Approximately 95% of all cervical cancers are caused by HPV infection and subsequent oncogenesis. In these types of cancer, HPV-derived expression of oncogenic proteins (i.e., the E5, E6 and E7 genes) results in the transformation of normal cervical cells. Thus the E6 and E7 are TAAs, potentially allowing for specific induction of immunological effector cells specific for HPV-transformed cervical epithelium. For example, He et al. created both vaccinia virus and [E1-]Ad-based vectors that both encoded the HPV E6 and E7 genes (13). These constructs were both tested for their ability to induce cancer immunity in both BALB/c and C57Bl/6 mice. The results of these studies demonstrated that depending on the strain of mouse model tested, either CD4+ or CD8+ T cells were stimulated by the viral vaccines. The class of immune effector cells elicited resulted in strikingly different responses to tumor cell challenge. The best results were obtained using the AdE7 vaccine in C57Bl/6 mice (~60% survival), a response found to be completely CD8+ dependent. No other vaccinations were successful in the C57Bl/6 mice. In the BALB/c mice, the AdE6 and VacE7 vaccines were equally efficacious (~40% survival), and both vectors induced CD4+-dependent responses. Though surprising, these results led the authors to conclude that [E1-]Ad vectors induce a more potent immune response compared to vaccinia virus-based vectors, and that a potent CD8 T-cell response was needed to eradicate HPV-16 tumor cells.

A similar study conducted using recombinant adenovirus encoding HPV-16 E5 found significant protection against tumor challenge after a single intramuscular injection of the vector into C57Bl/6 mice (14). Utilization of gene-specific knock-out mice confirmed that the effect was also CD8 T-cell dependent in these animals. Both studies reflect favorably upon the principle of using a recombinant Ad vaccination against particular/specific cancer genes, and that tumor rejection by CD8 T cells is possible once peripheral tolerance has been broken, although the studies suffer from the fact that the targeted TAA gene is also a potentially foreign antigen.

Another TAA target for cancer immunotherapy approaches is the prostate-specific antigen (PSA), a protein expressed only in prostate epithelial cells. Additionally, since this gene is normally expressed in healthy prostate epithelium, this antigen is a target that will induce auto-immunity against the entire prostate (which is, at some level, a non-essential organ). Since 95% of all prostate tumors overexpress PSA, it is a potent target for cancer vaccine strategies in general, and Ad vector-based cancer immunotherapy strategies specifically (15). In Ad vaccine studies involving this antigen, a PSA-specific T-cell response could be demonstrated in Ad-PSA-immunized mice (15). This Ad-PSA-induced immunity was both cellular and humoral, and could also protect mice against a subsequent subcutaneous challenge with PSA-expressing cancer cells. The antitumor immunity was predominantly mediated by CD8+ T lymphocytes, but this was only a protective immunity, as Ad5-PSA alone was unable to control growth of established, preexisting PSA tumors. Existing tumors (500–1000 mm³ in size) could only be eliminated if Ad5-PSA priming was followed 7 d later through an intratumoral injection of several canarypox viruses encoding different immunostimulatory factors (IL-12, IL-2, and TNF- α). In these instances, the immune response was dominated by CD8+ T cells, but natural killer (NK) cells were also necessary for effective tumoricide. Human phase I studies for specific prostate cancer antigens (prostate-specific membrane antigen [PSMA]) have yielded inconclusive results about the effectiveness of Ad vector PSA-

vaccination therapy (possibly because of subjects' concomitant hormone therapies and subject population heterogeneity), but have shown excellent safety profiles after viral administrations (16). Phase II trials to evaluate the efficacy are currently under way.

The specific immunoglobulin protein expressed by a B-cell lymphoma is another tumor-specific antigen, and as a self-antigen, is most likely a weak immunogen. In this, it is a specific and realistic target to test the efficacy of cancer vaccines in their ability to break tolerance and effectively target-specific cancer cells. In several studies, recombinant adenoviruses have been constructed that encode variable regions of the immunoglobulin light and heavy chains with or without a xenogenic Fc fragment (17–20). In vitro studies have shown cellular immunization with variable fragments alone can stimulate the production of T cells specific for the lymphoma (18). When taken to mouse models, the investigators were unable to demonstrate specific T-cell generation after injection of Ad vectors expressing variable region antigens alone (18,19). It was only after the variable regions were recombinantly linked to the xenogenic human Fc fragment (in the Ad vector construct) was the vaccination able to significantly inhibit tumor growth in mouse models. In these cases, a single immunization provided protection for over 40% of mice challenged with a lethal dose of the specific lymphoma line, a level of protection that was greater than could be previously achieved with optimized protein- or plasmid-based vaccines (18). When combined with chemotherapy, Ad vector vaccination significantly prolonged the survival of mice with preexisting tumor; however, vaccination alone wasn't sufficient to clear preexisting tumor loads (17). Again, these responses were noted to be largely CD8+ T-cell dependent, although significant antibody titers were also achieved against the targeted epitopes.

The antigen CO17-1A/GA733 has been consistently identified as a highly expressed antigen in various colorectal cancers and, as such, has been a useful target in both passive and active immunotherapy approaches using both monoclonal antibodies and anti-idiotypic antibodies. In addition to these strategies, the vaccination approach in targeting this human TAA has been tested using baculovirus, vaccinia, and Ad-based vectors to induce tumor-specific immunity (21). In that study, each of these human GAA733-expressing recombinant viruses induced antigen-specific antibodies and delayed-type hypersensitivity (DTH) responses in mouse models, but only the Ad vaccine was found to induce antigen-specific cytotoxic T lymphocytes (CTLs) and regression of established tumors. In a similar study using an Ad vector expressing human GA733, protected mice were also found to resist subsequent challenge with antigen-negative colorectal cancer tumors (the original tumors were antigen-positive colorectal cancer tumor lines), indicating that an immunologic crosslinking of tumor antigens after a successful vaccination could also be achieved (22). The results demonstrate the potency of successful Ad vaccination in colorectal cancer models, but were hampered by the fact that the recombinant Ad constructs encoded a xenogenic tumor gene (human GA733) delivered into a murine model system. When the murine homolog of the human GA733 antigen was used (murine epithelial glycoprotein, or mEGP) a recombinant Ad was unable to protect vaccinated mice against tumor challenge (21). Only when combined with IL-2 was recombinant Ad-mEGP able to significantly inhibit growth of established mEGP-positive tumors. Thus, although the significance of the immunological crosslinking in these studies cannot be overstated, use of an Ad-based vector alone did not appear sufficient to break native TAA tolerance unless xenogenes or other immunological stimulators (such as certain cytokines) were employed.

Another type of cancer presenting attractive targets for cancer vaccination approaches is melanoma. The melanosomal proteins TRP2, MART, and gp100 are all highly expressed in melanoma, and have been studied in experiments involving recombinant Ad vaccines. TRP2 is highly homologous in mice and humans, and although attempts at plasmid and gene gun vaccination have failed to induce immunity, administration of an Ad vector expressing a xenogenic human TRP2 caused mice to mount an immune response against their native melanocytes, resulting in coat depigmentation (23). These mice were also significantly protected against metastatic growth of B16 melanoma, an immunity associated with the presence of TRP2-reactive antibodies and CD8 T cells. In vitro studies of HLA-A2+ cell lines confirmed this association, in showing generation of melanoma-specific CD8 T cells for the other melanoma antigens, gp100 and MART-1 (24). Studies conducted in C57Bl/6 mice mirror results found with TRP2, showing a protective effect in mice vaccinated with melanoma tumor antigen (24). Furthermore, this protective effect is mediated principally through the generation of CD8+ T cells specific for the tumor antigen.

Based on this success, recombinant Ads encoding MART and gp100 were taken to human phase I trials. In these trials, 54 subjects with metastatic melanoma were given escalating doses (up to 1×10^{11} infectious units) of the Ad-based vectors with or without IL-2 (25). Of all the subjects receiving the Ad vector alone, only one achieved a complete response. Other subjects achieved their objective responses, but only with coadministration of IL-2. Immunological assays of subjects showed no consistent immunization to either MART-1 or gp100, although the authors concluded that this may have been attributable to the high level of neutralizing antibodies found in the subjects' sera prior to treatment, and/or the advanced state of the subjects' disease. Importantly, none of the escalating doses proved excessively toxic. The single clinical successes achieved in subjects through the coadministration of IL-2, demonstrate the potential of newer combined vaccine therapy approaches.

New vaccination strategies involve the ex vivo vaccination of a subjects' own dendritic cells (DCs) as a way to more effectively "educate" the immune system to target cancer antigens as well as minimize any possible side effects involved with administering high titers of recombinant adenovirus vectors directly in vivo. DCs are currently recognized as the key professional antigen-presenting cells (APCs) of the immune system and, as such, manipulation of DCs is a very active area of investigation in vaccine-based therapies. Antigen-loaded DCs efficiently interact with and mobilize the effector T cells of the immune system. These interactions are facilitated by DC coexpression of stimulatory adhesion molecules, and DC elaboration of cytokines such as IL-12 (26). Since one can generate clinically relevant numbers of DCs (i.e., from expansion of CD34+ progenitors in cord blood, bone marrow, or the peripheral circulation), the capability exists to "load" DCs with relevant proteins (typically tumor-specific antigens) ex vivo, and reinfuse the antigen-loaded DCs back into subjects to initiate and direct therapeutic immune responses (CTLs) to cells in the body expressing the antigen, i.e., cancer cells.

A number of methods are currently available to load DCs with antigens. Though several methods of DC transduction of DNA or RNA have been explored, many methods have been found to be significantly less efficient than similar attempts utilizing Ad vectors. For example, Arthur et al. demonstrated that Ad vectors encoding a variety of antigens could efficiently transduce 95% of the DC exposed to high titers of the vector

whereas Dietz et al. report that, when used in combination with liposomes, low titers of Ad vectors can effectively transduce more than 90% of exposed DCs (27). Importantly, increasing levels of gene expression were noted in the DCs with increasing multiplicities of infection (MOIs) with the Ad vector (28). It has been demonstrated that DCs infected with Ad vectors encoding a variety of antigens (including the tumor antigens MART-1, MAGE-A4, DF3/MUC1, p53, hugp100 melanoma antigen, polyoma virus middle-T antigen) have the ability to induce antigen-specific CTL responses, have an enhanced antigen presentation capacity, and have an improved ability to initiate T-cell proliferation in mixed lymphocyte reactions (27,29–35). Immunization of animals with DCs previously transduced by Ad vectors encoding tumor-specific antigens has been demonstrated to result in significant levels of protection for the animals when challenged with tumor cells expressing the respective antigen (36,37). Interestingly, intratumoral injection of Ads encoding IL-7 was less effective than injection of DCs transduced with IL-7 encoding Ad vectors at inducing antitumor immunity, further heightening the interest in ex vivo transduction of DCs by Ad vectors (38).

Ad vector capsid interaction with DCs (independent of Ad transcription) appears to trigger several responses, which may be enhancing the ability of DCs to present antigens. Controversy exists as to whether this effect is a result of an Ad-mediated induction of DC maturation (26,39). Studies of immature bone marrow-derived DCs from mice suggest that Ad vector infection of these cells resulted in upregulation of cell-surface markers normally associated with DC maturation (MHC I and II, CD40, CD80, CD86, and ICAM-1) as well as downregulation of Co11c, an integrin known to be downregulated upon myeloid DC maturation. In some instances, Ad vector infection triggers IL-12 production by DCs, a marker of DC maturation (26). These events may possibly be due to Ad-triggered activation of an NF- κ B pathway (40,41). Similar studies in mature CD83+ human DCs (derived from peripheral blood monocytes) demonstrated that mature human DCs were well transduced by Ad vectors, and did not lose their functional ability to stimulate the proliferation of naïve T cells when lower numbers of infectious units per DC were utilized; however, some studies suggest that mature DCs are less infectable than immature ones (39,42).

Furthermore, as DC transduction methods improve, DC abilities to stimulate antigen-specific T cells also improve. These studies suggest that, if one can improve tumor gene-encoded antigen expression from within DCs, one might improve the ability to generate T-cell-mediated immune responses. Unfortunately, at higher MOIs, DC cytotoxicity and DC death has been shown to be induced by [E1-]Ad vector infections, as well as causing a “blunted” ability of the mature DCs to stimulate naïve T cells, as compared to lower MOI infections of the mature DCs (42). The latter prompted the authors to suggest that residual gene expression derived from the [E1-]Ad vector backbone may have been the cause for this effect. This hypothesis has yet to be proven, but studies with fully deleted Ad vectors have suggested that residual gene expression may not contribute to DC activation induced by Ad vector infections (43–46). One trial currently under way involves the use of a recombinant Ad vector encoding MART-1 to transduce DCs ex vivo, and reinjecting these modified (“educated”) DCs as a potent cancer vaccine. This trial represents what will likely become the current and future thrust in Ad vaccine therapy approaches for cancer patients.

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Alphaviral-Based Strategies for the Immunotherapy of Cancer

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1. INTRODUCTION

Alphavirus-based vectors have been developed over the past 13 yr (1–3). Alphaviruses, formerly known as group A arboviruses, are small, enveloped, positive-strand RNA viruses that constitute one of two genera within the Togaviridae family (4,5). The alphaviruses infect a wide range of hosts being able to replicate in both arthropod and vertebrate hosts (avian and mammalian). The structure, biology, and replication strategy of these viruses have been well characterized over the past three decades (5,6). This characterization has resulted in the recognition of advantages and disadvantages for vectors derived from alphaviruses along with the identification of critical elements of the alphavirus genome that must be retained in the vector for various functional capabilities. The bulk of investigations using these vector systems have been focused on prophylactic vaccination against infectious-disease processes. Fewer studies have evaluated alphavirus vectors for their antitumor immunotherapy potential and all are preclinical. In contrast,

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Table 1
Alphaviruses Engineered for Vector Production

Semliki Forest Virus (SFV)
Sindbis Virus (SV)
Venezuelan Equine Encephalitis Virus (VEE)

DNA viruses, such as adenovirus or the pox viruses, have progressed to human cancer clinical trials, despite alphavirus vectors having distinct potential advantages. This is owing in part to studies with DNA viruses being initiated well before those of the alphaviruses along with the technical and regulatory demands of good manufacturing practices (GMP) production for RNA viruses. Three of the alphaviruses have been adapted to immunotherapeutic vectors and evaluated in various tumor models; *see* Table 1. The exact mechanisms by which these alphavirus-derived vectors elicit robust antitumor immune responses are unclear. However, it is likely that several of their characteristics contribute to their immunogenicity, including (a) the very high level of heterologous gene expression, (b) the vector-associated cytopathic effect, and (c) their specific cell and tissue tropisms.

2. OVERVIEW OF ALPHAVIRUS STRUCTURE AND BIOLOGY

There are over 25 identified members of the *alphavirus* genus, reviewed in references 4 and 5, many of which are geographically restricted despite the wide range of competent mosquito vectors and susceptible hosts. Alphaviruses are generally maintained in nature in cycles with different species of mosquitos, birds, or nonhuman mammals. However, viral zoonoses via mosquito vectors occur in humans with most of these viruses. These zoonotic infections can cause mild to significant morbidity and rare mortality in selected circumstances particularly in the very young and immunocompromised (5,7–13). Alphaviruses are capable of infection and proliferation in a wide range of tissues, including: neuronal, glial, skeletal and smooth muscle, synovial, epidermal, brown adipose, lymphoid, and other mesenchymal tissues (5,14,15). This diverse tropism may explain a number of human clinical symptoms including the nearly ubiquitous myalgia and arthralgia associated with these viral zoonoses along with the frequently observed headache/encephalitis and lymphopenia.

2.1. *Alphavirus Structure*

The structure and life cycle of representative alphaviruses have been extensively studied. Structurally, alphaviruses are relatively small viruses, approximately 60 nm in diameter. They consist of an icosahedral nucleocapsid accounting for approximately one-half of the diameter of the virion and an envelope that contains 80 glycoprotein “spikes” composed of the E1 and E2 glycoproteins. These spikes are comprised of three E1 and E2 heterodimers or heterotrimers (if E3 remains associated with the virion) arranged in a matching icosahedral lattice in a 1:1 ratio with the underlying capsid protein (16). The genomic RNA, ≈11–12 kb, is both capped and polyadenylated and represents a functional eukaryotic mRNA. Both the 5' and 3' ends contain short untranslated regions.

At least the 3' region appears to interact with host cellular proteins that are required for synthesis of viral negative strand RNA (5). The 5' approximately two-thirds of the genome, encodes the four nonstructural proteins responsible for RNA replication and transcription along with other functional capacities, and is translated in a single open reading frame (ORF) as a precursor protein that is subsequently cleaved into various functional subunits. The 3', approximately one-third of the viral genome, encodes the structural proteins and is also translated as a precursor protein that is cotranslationally and post-translationally cleaved to generate the capsid and mature envelope proteins. Viral infection of cells is glycoprotein spike dependent and small changes as subtle as a single site mutation can dramatically affect viral tropism and virulence (5,17,18).

2.2. *Alphavirus Infection Cycle*

The initial stage of an alphavirus infection uses receptor-mediated, charge-mediated, and endocytic uptake mechanisms in varying degrees depending upon the cell type, species, and alphavirus. Current evidence points to the existence of multiple receptors for alphaviruses. The putative protein receptors characterized to date are related to or identical with laminin receptors (19,20). Cellular uptake of alphaviruses and their derived vectors may also involve interactions with heparan sulfate or heparan-like molecules (21–24). Productive alphavirus infection requires transit of the virion through an acidic cellular compartment, endosome or lysosome, in order for successful envelope/membrane fusion and discharge of the nucleocapsid with subsequent release of the genomic RNA into the cytoplasm. The life cycle of the alphaviruses occurs entirely within the cytoplasm of infected cells. The genomic positive-strand RNA is translated directly for nonstructural protein synthesis, which is followed early in the infectious process by transient (only a matter of hours) synthesis of negative strand RNA (5,25). Thereafter, only positive-strand subgenomic and genomic RNAs are synthesized. The RNA replicase complexes are assembled on modified endosomal and lysosomal membranes (26). Virion production is noted approx 4 h after infection and reaches a plateau shortly thereafter. Cellular cytopathology is generally observed well before 24 h (6), although longer in some cases (27) with extended periods of cellular viability observed beyond 75 h with markedly attenuated viruses or viral vectors (28–34).

3. ALPHAVIRUS CHARACTERISTICS APPLICABLE TO VECTOR DEVELOPMENT

Alphaviruses have several characteristics that are potentially advantageous for the derivation of vaccine vector systems. Within the general population, preexisting anti-alphavirus immunity is not widespread. Alphaviruses generally exhibit diverse cellular tropism within a given host, allowing for a broad range of administration routes for antitumor immunotherapeutics, although some alphaviruses such as Venezuelan equine encephalitis (VEE) show a natural and pronounced initial tropism for lymphatic tissues and dendritic cells (35). In the case of single-cycle vectors, this tropism may be highly advantageous. The cytoplasmic life cycle of alphaviruses obviates transport across the nuclear membrane as a concern, in contrast to other DNA-based genetic (viral or plasmid) vaccines, and removes the potential for chromosomal integration or adventitial splicing of engineered coding sequences. The positive-sense polarity of alphavirus RNA genomes

results in the genomic RNA itself being infectious if transfected into susceptible cells in vitro or in vivo, as are RNA transcripts of full-length cDNA clones placed downstream of pol II promoters. Thus, both “naked” RNA and DNA vaccines based on replicating alphavirus RNAs are possible and have been assessed (6,36,37). The capped and polyadenylated genomic RNA allows for the efficient translation of viral RNA by mammalian host protein synthesis machinery. Indeed, these viruses rapidly co-opt host RNA and protein synthesis pathways resulting in very high levels of viral encoded protein synthesis, up to 25% of total cellular protein (1,2), providing abundant albeit transient expression of heterologous proteins. The cytopathic effect may play a significant role in the efficacy of elicited immune responses associated with alphavirus infections and alphavirus-derived vector systems.

These vector systems are well adapted to the high-level expression of “modified self” (38) tumor-associated antigens. The expression of the heterologous protein is intracellular and thus available to the endogenous antigen-processing and -presentation pathway. Furthermore, the associated cytopathic effect may also yield pro-inflammatory signals, facilitate dendritic cell (DC) “cross-priming,” and may be implicated in enhanced expression and loading of host heat shock proteins with peptides from expressed heterologous polypeptides. In several model systems, the cytopathic effect results primarily in apoptotic cell death (27,33,39–55). This cytopathic effect can result in uptake of heterologous protein contained in the cellular debris via the extrinsic antigen-processing pathway for presentation on major histocompatibility complex (MHC) class II and, in the case of DCs, also “cross-priming” into the MHC class I pathway. Although apoptotic cellular debris has been reported to result in DC-induced antigen-specific immunologic tolerance (56–58), controversy remains (59–72) and the effective application of these vectors in animal model systems does not support the development of immunologic tolerance. A very low level of preexisting immunity to the alphaviruses exists within the general population; thus, the clinical application of alphavirus derived vectors will not have to overcome this obstacle, in contrast to some of the other more commonly evaluated viral-based vector systems. In addition, as the structural proteins of the single-cycle alphavirus replicon vectors are not expressed in vaccine recipients, induction of antivector neutralizing immunity is minimal.

There are also potential disadvantages to the application of these viruses as vaccine vectors including their relatively small particle and genome size; with genomes approximately 50% that of adenovirus and 10% of pox viruses (73). This limits the “loading capacity” for heterologous coding sequences able to be inserted into the vector, although inserts up to 7 kb have been efficiently translated. An additional potential drawback for replication-competent RNA viruses, including the alphaviruses, is the relatively low fidelity of the replication process, estimated to be 2–4 logs lower than the fidelity of DNA replication, with an estimated error rate for RNA-directed RNA synthesis of one misincorporation in 10^4 – 10^5 bases (73). Additionally, as noted below there is a theoretical risk of RNA recombination among vector and helper RNAs in the cell cultures used for particle assembly yielding replication-competent virions with the associated risk of illness in immunocompromised hosts such as cancer patients.

4. ALPHAVIRUS REPLICONS AND RELATED VECTOR SYSTEMS: GENERAL CHARACTERISTICS

Some efforts have been made to generate vaccine vectors from fully replication-competent alphaviruses. In these cases, attenuated strains are used, and the subgenomic 26S promoter is duplicated to allow expression of both the viral structural proteins and a heterologous gene (74). However, such vectors are limited with respect to both the size and stability of the heterologous gene, and in addition, efficiently induce antivector immune responses. Thus, replicon vectors have proven to be more flexible, and have significant advantages in terms of potential safety and loading capacity.

In the design of alphavirus replicon vectors, the coding sequence for the structural proteins is replaced completely or in part by the coding sequence for heterologous protein(s). Because the packaged RNA within these alphavirus vectors retains the capacity to synthesize the usual abundance of complementary full-length negative-strand RNAs, subgenomic positive RNAs, and full-length genomic positive-strand RNAs, these vectors are frequently referred to as “replicons.” These replicon RNAs are packaged into virus-like particles by providing the structural protein genes “*in trans*” on other (“helper”) RNA or DNA molecules. Typically, packaging signals are excluded from the helper elements, such that only the replicon RNA is packaged. This amplification of vector-derived RNA is key to the abundant expression of heterologous proteins and may play an indirect role in the cytopathic effect. A number of *cis*-acting elements have been defined that are critical for this RNA replication and heterologous protein expression in permissive cells (2,3,75–78). Obviously, in the absence of the requisite structural proteins infectious virions are not produced, and therefore replicon systems constructed in this manner are single-cycle vectors, capable of one round of cell infection but not capable of systemic spread or multiple rounds of infection. RNA amplification and gene expression remain intact; thus, the term “replication incompetent viral vectors” has to be applied with caution to alphavirus replicons. Importantly, the safety of these vectors is provided by genetic restriction, lacking the genetic elements necessary for structural protein synthesis, and is not dependent upon any immune response from the vaccinee to ensure safety.

4.1. *Replicons*

Several methods for the production of alphavirus replicon vectors have been described. The most efficient expression of heterologous proteins from replicons is accomplished when the desired ORF is engineered into the approx 4-kb 3' region encoding the structural proteins and downstream of the subgenomic viral promoter, which provides a 10-fold amplification of message. This derivative has been described for all three of the alphavirus vectors under consideration in this chapter, Semliki Forest virus (SFV) (2,75), Sindbis virus (SV) (3,76), and VEE virus (1,74). To package these engineered replicon RNAs, the structural protein genes must be provided *in trans*. This can be accomplished by providing competent helper virus that results in virions containing either the engineered or wild-type genomic RNA, as demonstrated by Xiong et al. with Sindbis vectors (3). Alternatively, a deleted form of the genomic RNA, with an incomplete nonstructural protein gene cassette and all packaging signals removed, has been used to provide for translation of structural proteins *in trans* for SFV- (2,76), SV- (75), and VEE-based vectors (1). For SFV and SV, such systems are commercially available, SFV Gene

Expression System (Gibco BRL Life Technologies, Grand Island, NY) and Sindbis Expression System (Invitrogen Corp., Carlsbad, CA), respectively. However, due to the relatively high frequency of recombination between replicon and single RNA helpers (estimated 10^{-3} to 10^{-6}), such single-helper configurations often lead to the generation of replication-competent virus (79,80). As all of these viruses can be human pathogens (5,7–13), improved packaging strategies that make use of two separate defective helper RNAs (split-helper systems), one encoding the capsid protein and the other the envelope glycoproteins, have been described (1,6). In this strategy, a minimum of two separate recombination events would need to occur to generate a replication-competent virus. More recently, a similar split-helper RNA system for SFV has been described (81). Traditional packaging cell lines for SFV- and SV-based vectors have also been described (82). Attenuating mutations have also been incorporated into SFV- and VEE-based vector systems. The relatively high frequency of reversion due to RNA replication infidelity, estimated at 10^{-4} – 10^{-5} , suggests that several methods need to be employed to reasonably assure that wild-type virus does not contaminate replicon preparations. As VEE is a biosafety level (BL)3 agent in the United States, cotransfection with VEE-based replicon and helper elements require BL3 production facilities. Although SFV itself is also a BL3 virus, some SFV vector systems have been approved for use at BL2.

4.2. Hybrid Vectors

Several groups are evaluating hybrid DNA vaccine and alphavirus replicon technologies (83–89). These systems take advantage of standard DNA plasmid production systems in lieu of the more technically demanding replicon particle production methods. The plasmid constructs use typical promoters active in eukaryotes to drive transcription of positive-strand RNA from the plasmid, via an RNA polymerase II-dependent process. This RNA transcript represents the engineered genomic alphavirus RNA missing the coding region for the structural proteins and containing coding sequences for heterologous protein(s). This RNA then functions in a manner identical to the engineered genomic RNA introduced via transfection or replicon particles as described above. Although production and ease of handling are distinct advantages of this hybrid technology, the plasmid needs to be translocated across the nuclear membrane and the transcribed genomic RNA similarly transported out of the nucleus. Thus, this hybrid technology abrogates one of the advantages of the alphavirus systems. Additionally, unlike viral-like particles, DNA vaccines do not have cellular tropism and are not yet able to be targeted to specific cell types. Because these strategies essentially deliver engineered alphavirus genomic RNA via plasmids, they will be included for discussion in this chapter, as will the reports of direct polynucleotide immunization with engineered genomic alphavirus RNA.

5. SEMLIKI FOREST VIRUS-DERIVED VECTORS

The SFV replicon system was described by Liljestrom and Garoff in 1991 (2). Two plasmids, one encoding the four nonstructural proteins, the subgenomic promoter and a cloning site for insertion of the coding sequence for a heterologous protein, and the second, a helper plasmid encoding the structural proteins, are transcribed in vitro using the SP6 promoter and RNA polymerase. The resultant RNAs are then transfected by electroporation or lipofection into permissive cells, most frequently BHK-21 cells (CCL10,

Table 2
SFV-Based Antitumor Vaccines

Reference	Antigen/Tumor	Tumor Model	Immune Responses
107	P1A/P815A	Protection	CTL
108	P1A/P815A	Treatment	Not characterized
83	E7-HSP70/TC-1	Protection	Th1 cytokines, CD8 dependent
		Treatment	
95	E6-E7/TC-1	Protection	CTL
96	E6-E7/TC-1	Protection	CTL, IFN- γ ELISPOT
110	Id Ig/lymphoma	In vitro human	CTL
54	cDNA/203 glioma	Protection	CTL
	B16	Treatment	
112	LacZ/multiple	Protection	CTL, Antibody
111	LacZ/CT26	Protection	Antibody
		Treatment	IFN- γ production
85	LacZ / CT26	Protection	Antibody
			IFN- γ production

ATCC, Manassas, VA). Yields of up to 5×10^7 replicons/mL are obtained with packaging reactions of 10^7 cells from the commercial system, although higher titers have been reported (2,90). With the BL2 system, the p62 glycoprotein spike has been mutated to resist the normal late cleavage into E2 and E3 glycoproteins, which is required for infectivity of the SFV replicon. This infectivity can be restored by treatment of the replicon particles in vitro with α -chymotrypsin A4 at a final concentration of 200 μ g/mL. The host tissue receptivity for SFV replicons includes skeletal muscle and lymphoid tissue (91), epidermal cells (15), peritoneal epithelium (92), smooth muscle, and cardiomyocytes (93).

SFV replicons have been used in a number of model systems to elicit or enhance antitumor immunity; see Table 2. Some efforts have made use of the robust expression of heterologous proteins in strategies that are more closely related to gene therapy, e.g., cytokine-transduced tumors or local cytokine production (33,92,94), and will not be discussed in this chapter. Others have used SFV-mediated cytopathic effect for eliciting antitumor effects (49,50) and similarly will not be discussed here.

5.1. HPV

The human papilloma virus (HPV) is intimately associated with cervical cancer and the HPV E6 and E7 proteins are attractive tumor-associated antigens (TAAs) present in the overwhelming majority of cervical cancers. Daemen and colleagues have targeted these two proteins in two separate reports, initially as two separately expressed full-length proteins (95) and subsequently as a fusion protein (96). Pulse chase experiments suggested that the individually expressed heterologous E6 and E7 proteins were essentially 100% degraded by 6 h whereas the fusion protein was relatively stable with an apparent $T_{1/2}$ of between 6 and 10 h. Using SV replicons, this group was able to elicit antigen-specific cytotoxic T lymphocyte (CTL) responses to 13–2 cells (expressing an E7 CTL epitope AA49-57 RAHYNIVTF) or C3 cells (containing the complete HPV-16 genome)

and significant protection to TC-1 tumor challenge, a ras-transformed HPV-16 E6E7 expressing primary lung epithelial cell line derived from C57BL/6 mice. The CTL responses were identified in bulk cultures with limited restimulation (one to two stimulations at 7-d intervals). These responses required administration of at least 10^6 SFV replicon particles. In an effort to assess a dose-response relationship, this group placed an enhancer upstream of the fusion construct with subsequent demonstration of CTL activity with 10^5 SFV particles as the immunogen. Similarly, detectable interferon- γ (IFN- γ) ELISPOT activity was only seen in splenocytes of mice immunized with the construct including the enhancer element. In vivo tumor challenge experiments recapitulated the in vitro immunologic assays with the enhancer fusion construct demonstrating the best efficacy.

The group of T-C Wu have examined the role of *Mycobacterium tuberculosis* HSP 70 linked to the HPV E7 antigen (83,97). They modified their previously described conventional DNA vaccination strategy to include a “suicidal DNA” construct that uses the CMV-IE promoter to drive initial transcription of an engineered SFV replicon RNA expressing the HPV E7 antigen. These “hybrid” plasmid constructs were administered by gene gun. Although there were no differences in IFN- γ or IL-4 expression in CD4 T cells, the fusion protein construct resulted in a significantly enhanced intracellular cytokine staining for IFN- γ in CD8 T cells. This construct was the only one that provided protection in tumor challenge experiments and demonstrated decreased pulmonary metastases in a treatment model.

The data of Daemen and colleagues would seem to be inconsistent with data suggesting that MHC class I antigen presentation is enhanced with more rapid degradation of target antigen proteins (98–103). However, if antigen hand off to antigen-presenting cells (APCs) occurs, then the persistence of antigen would be advantageous. One might also argue that the fusion protein is more likely to be misfolded than the independently expressed E6 and E7 proteins. Malfolded proteins are more likely to associate with heat shock proteins (HSPs) and other chaperones and may be handled somewhat differently than normal with respect to degradation and entry into the antigen presentation pathway (104). This may contribute to the enhanced immune responses observed by this group. In this context, it is worthwhile to note that the use of SFV to enhance in vitro production of HSP peptide complexes for subsequent protein-based immunization strategies has been reported (105). Thus, it seems that SFV, in and of itself, can upregulate HSP expression, further complicating the mechanistic dissection of the enhanced immune responses seen in the above reports.

5.2. P815 Mastocytoma

The P1A gene encodes a potent TAA for the P815 murine mastocytoma tumor (106) as described by Boon and colleagues. Colmenero and colleagues have targeted this antigen with and without interleukin (IL)-12 (107,108) using SFV replicon particles. They detected increasing specific CTL responses that reached a plateau at a 10^6 IU SFV replicon dose and were long-lasting (up to 100 d post last immunization). This was associated with protection from tumor challenge in 70–85% of animals with a construct containing a translational enhancer, consisting of a coding sequence for 34 amino acids from the capsid protein, showing slightly better efficacy despite essentially identical CTL responses. Antibody responses to P815 A or B antigens were not detected. In the follow-

up study, they evaluated peri-tumoral injection in a treatment model and demonstrated approx 50% tumor regression and no statistical difference when combined with an IL-12-producing SFV replicon (108). This group also examined route of administration using non-TAA (109). Intravenous (iv) administration of SFV replicons gave maximal numbers of tetramer-positive splenocytes, but the least number of tetramer-positive cells in draining lymph nodes. Subcutaneous (sc) administration yielded the exact opposite result and intramuscular (im) administration was nearly as effective as iv in generating tetramer-positive splenocytes and clearly intermediate with respect to lymph node tissue. In two of three experiments, there was no statistical significance between iv- or im-induced CTL responses.

5.3. Idiotypic Immunoglobulin

Osterroth and colleagues performed in vitro pilot studies with molecular rescues of B-cell non-Hodgkin's lymphoma idiotypic immunoglobulin heavy and light chains from two separate patients. They evaluated the capacity to induce idiotype-specific CTL via stimulation of autologous lymphocytes with autologous, monocyte-derived DCs loaded via SFV replicons expressing the idiotypic immunoglobulin as the heterologous protein (110). These coding sequences for the unique TAA, idiotypic immunoglobulin, were subcloned into a standard eukaryotic expression plasmid and also into the SFV replicon constructs. Either recombinant protein or SFV replicons encoding the idiotypic immunoglobulin were incubated with autologous monocyte-derived Langerhans cell-type DCs prior to use for in vitro generation of CTL over a minimum of three weekly stimulations. Stable transfectants of autologous B lymphoblastoid cell lines (LCLs) expressing idiotypic immunoglobulin were the targets for assaying CTL activity. Despite SFV-induced expression of idiotypic immunoglobulin in lipopolysaccharide (LPS)-matured DCs, no idiotypic immunoglobulin-specific CTL activity was observed, leading the authors to conclude that direct SFV transfection of DCs was less efficient than coincubation with recombinant protein in this regard (110). These data suggest that despite the capacity for SFV replicons to transfect Langerhans-type DCs, the antigen processing and presentation of heterologous protein is not particularly efficient.

5.4. Glioma and Melanoma

Yamanaka and colleagues have used SFV replicons for multiantigen immunotherapy in the 203 glioma and B16 melanoma models derived from C57BL/6 mice (54). Tumor cDNA was generated from tumor mRNA and cloned into the SFV replicon constructs yielding "SFVcDNA-RNA" (54). This mixture of SFV replicons expressing a wide array of tumor-derived heterologous proteins was incubated with syngeneic, Day 6, bone marrow-derived DCs that were then used as the immunogen via three intraperitoneal injections at 1-wk intervals. Splenocytes from immunized animals were evaluated for CTL responses and the SFV replicon-loaded DCs yielded substantial tumor-specific CTL responses, approximately twofold greater specific cytotoxicity at each effector to target ratio compared to splenocytes from animals immunized with tumor RNA or tumor lysate-pulsed DCs. In tumor protection models, tumor outgrowth was delayed with the SFV-cDNA replicon-loaded DC immunizations and 50% full protection was demonstrated using intracranial challenges of either 203 or B16 with the respective matched SFV cDNA replicon-loaded DC immunization. In a tumor treatment model, 500 tumor cells

were implanted in the brains of mice with initiation of the immunization sequence 4 d later. Fifty percent of the SFV-cDNA-loaded DC-immunized animals were long-term survivors (90 d) for each tumor. No other immunization condition resulted in long-term survival of any animals. This group demonstrated that SFV replicon-transfected DCs underwent apoptotic cell death with less than 20% of the cells in culture remaining viable after 96 h and further that these apoptotic cells were taken up efficiently by unmanipulated immature DCs in vitro. The discrepancy between these results and those of Osterroth and colleagues described above may reflect the different phenotypic subsets of DCs used by each group.

5.5. *LacZ*

Several groups have utilized the *LacZ* model antigen system for evaluating various SFV-derived antitumor immunization strategies. The group of N. Restifo has used direct polynucleotide immunization strategies (85,111). The first report evaluated an in vitro-transcribed, replicon RNA-engineered to express *LacZ*. This RNA was injected im either 21 d before tumor challenge or starting 2 d after tumor inoculation. Antibody responses and IFN- γ production were demonstrated with the replicon RNA vaccination. In a dose-dependent manner, prevention of pulmonary metastases from CT26.CL25 *LacZ*-expressing tumor cell line was demonstrated. In their treatment model, again with CT26.CL25, delayed tumor outgrowth was demonstrated and when combined with IL-12 approx 40% of animals survived 60 d (111). The capacity to elicit these immune and clinical responses was associated with the degree of BHK apoptosis induced by each RNA or DNA construct. A similar direct polynucleotide immunization strategy was used by Schirrmacher and colleagues to demonstrate the superiority of self-replicating RNA, engineered SFV genomic RNA, over nonreplicating RNA and DNA expression constructs with respect to antibody, tumor challenge protection, and *LacZ*-specific CTL induction (112). The Restifo group also evaluated a “hybrid” strategy in the same model system as used for the replicon RNA immunization studies (85). Here a CMV-IE promoter was used to drive initial transcription of two different alphavirus, SFV and SV, engineered replicon RNAs from a standard DNA expression plasmid. The SFV construct was essentially identical in content to that evaluated in the earlier study of transcribed SFV replicon RNA immunization (111). The SV construct was essentially identical in design, only derived from the heterologous alphavirus SV (85). Significantly higher antibody responses and bulk IFN- γ production were reported with the replication-competent “hybrid” constructs than with the two standard expression plasmids using the CMV promoter to directly drive expression of β -Gal. Interestingly, the SV construct showed an inverse dose response relationship with bulk IFN- γ production and better tumor protection relative to the SFV construct or the standard expression plasmids. The SV construct provided delayed tumor progression in the treatment model. These differences were not due to level of antigen expression as measured in BHK cells. Indeed, the least immunogenic construct produced the highest percentage of total cellular protein as β -Gal and the SV construct produced the least after adjusting for transfection efficiency. The cytopathic effect of both SFV and SV constructs was identical at 24 and 48 h with the SFV construct having slightly more cytopathic effect at 72 h. The characteristics that account for the enhanced efficacy of the SV vs SFV construct in this model system remain unknown.

Table 3
SV-Based Antitumor Vaccines

<i>Reference</i>	<i>Antigen/Tumor</i>	<i>Tumor Model</i>	<i>Immune Responses</i>
123	E7*/TC-1	Protection	CTL, bulk IFN- γ production, NK, CD4, CD8 dependent
97	E7-HSP70/TC-1	Protection	CTL, bulk IFN- γ production, CD8, NK dependent
125,126	E7-VP22/TC-1	Protection Treatment	CTL, bulk IFN- γ production, CD8, NK dependent
127	Neu/66.3 and P815 stable transfectants	Protection	Bulk IFN- γ production
85	<i>LacZ</i> /CT26	Protection Treatment	Antibody IFN- γ production

6. SINDBIS VIRUS-DERIVED VECTORS

SV and derived SV vectors have been extensively studied in the United States. The SV replicon system was the first of the three alphavirus replicon systems to be reported, in 1989 by Xiong et al. (3). As originally described, the engineered SV replicon RNA was packaged by transfection of in vitro-transcribed RNA into BHK cells that were then infected with wild-type SV. As the replicon RNA contained the packaging signal, it was packaged in a small percentage of virions essentially competing with wild-type SV genomic RNA for encapsidation and assembly into infectious particles. The efficiency of producing particles containing replicon RNA encoding heterologous protein was estimated at approx 10%. A packaging system analogous to that described above for SFV replicons utilizing two in vitro-transcribed RNAs, one encoding the structural proteins and the other the engineered “genomic” RNA, was described in 1993 (76). However, this system still resulted in significant contamination of the replicon preparations with replication-competent virions capable of propagating an active SV infection (79,113,114). Use of nonredundant split RNA helper system, as used for VEE replicons, was described in 1997 but not used for antitumor immunotherapy (115). The host tissue receptivity for SV replicons includes fibroblasts and skeletal muscle (6), neuronal cells (116), and cardiomyocytes (31). In contrast to SFV and VEE, SV does not have significant tropism for lymphoid tissues unless mutations are introduced into the envelope glycoproteins (117–121). Comparisons of various alphavirus-derived expression systems demonstrate SV to have equivalent or more robust heterologous protein expression, compared to SFV (85,114,118,122), and SV may have other as yet undefined characteristics that result in more robust antitumor immune responses (85). SV replicon RNA-based polynucleotide immunization or derived hybrid polynucleotide vectors have been or are being evaluated for the capacity to elicit or enhance antitumor immunity in four model systems (*see* Table 3), with multiple modifications in one model system. SV replicons *per se* have not been reported in antitumor immunotherapeutic strategies. One model system, the *LacZ* model antigen, has been discussed above (85). Work in one model system, CEA, was funded by NIH in 2001 (Conroy, R.M., University of Alabama, CRISP Database), but there have

been no published reports or presentations on this work to date, thus it will not be discussed further.

6.1. HPV

The group of T.C. Wu has evaluated three modifications to the E7 antigen from HPV-16 in the context of SV replicon RNA constructs administered as RNA polynucleotide immunizations. The first modification involved construction of two fusion protein constructs, the first with an endoplasmic reticular (ER) translocation signal sequence fused to E7, and the second consisting of the first construct fused to the transmembrane and lysosomal targeting domains derived from the lysosomal-associated membrane protein-1 (LAMP-1) (123). The addition of the ER signal sequence did not result in any advantage, however the dual-fusion construct resulted in a four- to sixfold decrease in the number of pulmonary metastatic nodules in a dose-dependent fashion to a maximum dose of 10 µg RNA. This was associated with maximal bulk IFN- γ production in response to splenocyte incubation with two separate peptide epitopes contained within the E7 protein and significantly increased anti-TC-1 CTL responses after a single, 1 wk stimulation with E7 peptide. Antibody responses were not significantly different between the three constructs. Evaluation of BHK cytopathic effect for these various constructs revealed no significant difference. Interestingly, antibody depletion studies supported a very significant natural killer (NK) component to the antitumor immune response although, combined CD4 and CD8 depletion resulted in nearly the same number of pulmonary metastatic nodules as naïve animals. In a manner analogous to that of Albert et al. (72,124), Chen and colleagues evaluated the capacity of murine bone marrow-derived DCs to present E7 obtained from apoptotic SV replicon-transfected BHK cells on MHC class I with resultant cytosis by antigen-specific CTL. Only the dual construct resulted in significant specific cytosis, suggesting that this construct provided, in some way, for enhanced DC “cross-priming.”

The second modification evaluated by the Wu group consisted of a fusion protein with *M. tuberculosis* HSP70 and E7 incorporated into SV replicon RNA backbone (97). This fusion construct model when administered as an RNA polynucleotide immunization provided a 4 to >10-fold improvement in the number of pulmonary metastatic nodules in the TC-1 challenge relative to an unmodified E7 construct or other control animals. Similarly, they demonstrated enhanced CTL responses, bulk IFN- γ production, and cytosis of DCs loaded with apoptotic, SV fusion construct-transfected BHK cells, all as determined in the preceding study. Antibody depletion studies failed to demonstrate a significant role for CD4 T lymphocytes and NK cells remained the dominant effector cells. Although not compared directly, the data from the combined studies performed by this group suggest that the HSP-70 target antigen fusion construct was superior to the ER and LAMP fusion constructs. Within these various studies, the results for control constructs were comparable, however the magnitude of the CTL responses along with the decrease in pulmonary metastatic nodules were all larger with the HSP-70 target antigen fusion construct (97,123).

The third modification of the E7 target antigen evaluated by this group was a fusion with the herpes simplex VP22 tegument protein (125,126). The VP22 protein has been reported to mediate transmembrane migration of proteins, thus allowing for the distribution of these proteins to “bystander” cells. Essentially identical studies were performed

to those described above (125). Again, an approx 10-fold maximal decrease in the number of pulmonary metastatic nodules was reported with immunization by the VP22/E7 fusion SV self-replicating RNA which was associated with significant (although not as robust as in the HSP70 fusion studies [97]) TC-1 CTL responses, bulk IFN- γ production, and cytolysis of DCs loaded with apoptotic transfected BHK cells. In this study, antibody depletion experiments demonstrated that the CD8 T cells were the dominant effector arm, although NK cells were also very active. The companion report (126) demonstrated that this VP22/E7 SV self-replicating RNA immunization resulted in enhanced numbers of CD8 T splenocytes with positive IFN- γ intracellular staining in response to incubation with E7 peptide and similar benefits to a subcutaneous TC-1 tumor challenge. They also report a significant benefit in a treatment model, an approximate 2/3 decrease in the number of pulmonary nodules, associated with the administration of SV self-replicating RNA on d 3, 7, and 14 after iv tumor inoculation. Histologic assessment of the immunization site demonstrated that the VP22/E7 SV immunization resulted in a greater degree of myocyte apoptosis than the other SV self-replicating RNA (126). This same difference was not noted in the earlier report (125). It seems unlikely that this level of increased skeletal muscle apoptosis can account for the observed benefit of this modification of the E7 tumor-associated antigen. Especially, given that there were no significant differences in BHK apoptosis using these various constructs and the previous demonstration by this group that SV self-replicating RNA constructs with similar levels of apoptosis yielded different efficiencies of DC “cross-priming.”

6.2. *Neu*

Lachman and colleagues (127) targeted rat *neu* in two murine models including a rat *neu* transgenic model using the ELVIS plasmid (128). This is a hybrid vector system, derived from SV, using the CMV promoter to drive RNA polymerase II dependent transcription of a self-replicating positive-strand genomic-length RNA. This strategy is comparable to those discussed above (83,85). Using a stable transfected rat *neu* cell line derived from the syngeneic tumor-cell line 66.3, protection from tumor challenge was evaluated after im or id polynucleotide injections. Single immunizations resulted in delayed tumor outgrowth but no protection. Three immunizations occurring every 2 wk resulted in protection of 75% of the mice from subcutaneous tumor challenge and decreased the number of pulmonary metastatic nodules in an iv tumor challenge. Similar immunization of FVB/N rat *neu* transgenic mice demonstrated significant delay, 40 d, in development of spontaneous mammary tumors relative to nonimmunized animals. A single immunization yielded bulk IFN- γ production by splenocytes incubated with the irradiated rat *neu* transgenic cell line and no detectable increase in IL-4 production over background.

7. VENEZUELAN EQUINE ENCEPHALITIS REPLICONS

Alphavirus replicon vectors derived from VEE virus have also been recently applied to antitumor immunotherapy. The original report of the split helper RNA production system for VEE virus replicon particles (VRPs) was published in 1997 (1). In addition to the use of two separate nonoverlapping helper RNAs, the coding sequences for the various structural genes have incorporated previously described attenuating mutations (74,129). To some extent, the BL3 restriction has limited the number of groups investi-

Table 4
VEE-Based Antitumor Vaccines

<i>Reference</i>	<i>Antigen/Tumor</i>	<i>Tumor Model</i>	<i>Immune Responses</i>
131,132	PSMA	Protection	IFN- γ ELISPOT
130	E7/C3	Protection Treatment	CTL, bulk IFN- γ production

gating this alphavirus and its derived vector systems. Currently, reagents for generating alphavirus replicons are not commercially available, however AlphaVax, a biotechnology company in Research Triangle, NC, is commercializing this technology. The host tissue receptivity for VEE and derived replicons includes lymphoid and neuronal cells, and specific targeting to DCs has been described (5,35). This tropism is in keeping with clinical observations in humans in which the initial tissues infected are lymphoid. It is this innate tropism for lymphoid tissues that has spurred interest in VEE-derived vectors. There have been no reports of VEE-derived “hybrid” polynucleotide vectors. Evaluation of VRPs for antitumor immunotherapy has been reported in two model systems; *see* Table 4.

7.1. HPV

Velders and colleagues evaluated full-length unmodified E7 as the target antigen in a VRP subcutaneous immunization strategy using an individual dose of 3×10^5 IU (130). This group also used the C57BL/6 syngeneic C3 tumor cell line expressing HPV-16 E7 rather than the TC-1 line used with the previously described HPV studies evaluating SV- and SFV-derived systems. This VRP immunization regimen provided 100% protection against the tumor challenge. This protection was associated with (a) detection of 5.7% antigen-educated CD8+ splenocytes by tetramer staining, using the E7_{49–57} peptide, (b) a 10-fold increase over background and irrelevant peptide incubation in IFN- γ ELISPOT signal in response to incubation of splenocytes with the E7_{49–57} peptide, and (c) increased E7-specific CTL responses. CD4 and CD8 knockout animals were used to demonstrate the critical role for CD8+ cells. An experiment with a delayed tumor challenge, 90 d after completing the immunization sequence, demonstrated no degradation of the antitumor immune responses, as again, 100% of the animals were protected from the tumor challenge. Evaluation of the E7-containing VRP immunization strategy in a 1 wk, palpable tumor nodule, treatment model resulted in regression of tumor in 67% of the animals at 60 d. This strategy did not require modification of E7 as described in the experiments using SV-derived self-replicating RNA polynucleotide immunization strategies (97,123,125,126).

7.2. Prostate Cancer

Prostate-specific membrane antigen (PSMA) is a glycoprotein normally located within the intracellular compartment in normal prostate epithelium. However, upon neoplastic transformation, this protein exists as a type II membrane protein. The work using VRPs as a vector system targeting PSMA has been reported in abstract form only (131,132). The full-length PSMA-coding sequence was used for heterologous protein expression in the

VRP system. Mice were immunized with VRPs designed to express this xenoantigen and both antibody and cellular immune responses were identified. Both responses were reflective of a Th1 bias to the elicited immune responses as characterized by cytokine production, IFN- γ and IL-4, for CD4 and CD8 T cells by ELISPOT and murine antibody isotype.

7.3. Neu

We have recently embarked upon investigations targeting the rat neu protein. The target antigen is not the full-length molecule and has been designed to elicit primarily a cellular, Th1, and CTL immune response in lieu of a humoral immune response. The animal model system is a syngeneic rat mammary tumor model that moderately overexpresses rat neu. In this very rigorous mammary tumor model, im administration of VRPs encoding the target antigen provides protection from tumor challenge, in a dose-dependent manner, reaching 50% at a dose of 10^7 IU and immunologic memory is established. Additional studies are under way to fully characterize the antitumor immune response in this system.

8. CONCLUSIONS

The biology of alphaviruses and the experience with alphavirus vectors to date suggests that they may be particularly well suited for adaptation to antitumor immunotherapy. Their self-replicating positive-strand RNA, resultant robust heterologous protein expression, and tissue tropism are likely to prove to be advantageous. However questions remain regarding optimal antigen dose, route of immunization, and context of antigen expression with respect to “danger signals.” It remains to be seen which of the alphavirus-derived strategies will be most effective. Recent investigations have targeted cervical cancer (HPV-16), hematologic neoplasms (P815 and non-Hodgkin’s lymphoma), melanoma, glioma, prostatic adenocarcinoma, neu-expressing tumors, carcinoembryonic antigen (CEA)-expressing tumors, and the *LacZ* model antigen-expressing tumor systems. Of these, significant tumor treatment effects have been demonstrated in the HPV, glioma, melanoma, P815, and *LacZ* models. The capacity to demonstrate treatment efficacy suggests that these vector systems will prove to be valuable additions to the immunotherapeutic armamentarium and may well prove to be clinically beneficial in human clinical trials. More fundamental laboratory mechanistic studies and preclinical studies will be needed both to address the many remaining questions and to lay a foundation for the clinical investigation of these promising vectors in human clinical trials.

9. FUTURE DIRECTIONS

The efficient application of alphavirus-derived antitumor immunotherapeutic strategies will rest upon an increased appreciation of the subtleties of both fundamental immunology, particularly the role and biology of DCs, and the interaction of host tissues with alphavirus-derived vectors. The role of alphavirus-induced cytopathic effect is not clear, as demonstrated in the studies discussed above, which report varying degrees of correlation between cytopathic effect and elicited antitumor immune responses. The recent description of alphaviruses (30,32) with reduced cytopathology and their derived vectors should allow for adequate dissection of this fundamental question. Recent reports suggest that the alphavirus cytopathic effect is not solely mediated by replication (46) and may be mediated directly by envelope glycoproteins (133); although, the number of glycopro-

tein molecules introduced in the transfection of host cells are very limited and may not be sufficient to play a role in the cytopathic effect. However, understanding the role of envelope glycoproteins with respect to cellular tropism may lead to the design of more effective alphavirus replicon vectors. The effectiveness of hybrid polynucleotide strategies suggests that optimizing envelope glycoprotein structure and function may be desirable, but perhaps not required for effective antitumor immunotherapy.

All alphavirus-derived vectors, described above, yield abundant intracellular expression of heterologous proteins. However, the optimal level of expression and duration of expression for any one antigen, let alone multiple TAAs, remains unknown. The studies described above that report conflicting data with respect to increased antigen dose (85,96) support the view that each antigen and model system is likely to have its own optimal parameters. The reported high levels of protein expression and associated Th1 immune responses seen with these alphavirus-derived strategies are in agreement with the observations of Langenkamp and colleagues that DCs exposed to high doses of antigen elicit Th1-biased immune responses (134). Whether alphaviruses or derived replicon vectors trigger the innate immune response via pathogen-associated molecular pattern (PAMP) receptors such as the Toll-like receptors (TLRs), e.g., TLR-3 and dsRNA (135), remains poorly understood. The conflicting results of Osterroth (110) and Yamanaka (54) with respect to SFV replicon loading of DCs may be related to the use of different DC subsets. This suggests that characterizing the interaction between the various subsets of DCs and both alphavirus-derived vectors and/or primary transduced cells, including downstream events, will likely impact not only the design of antitumor immunotherapeutic strategies but also shed light on fundamental aspects of DC biology. It is likely that progress in understanding these fundamental immunologic issues will parallel the progress in developing efficacious antitumor immunotherapeutics.

Finally, the recent development and description of small interfering RNA-based interventions (136–141) suggests new avenues of investigation for the application of alphavirus-derived vector systems. Alphavirus delivery of ribozymes has been described previously (142). The development of noncytopathic vectors and the increasing understanding of alphavirus tropism and replicon targeting will undoubtedly aid these endeavors and, further, add to the enormous therapeutic potential of alphavirus-based immunotherapeutic strategies.

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16

DNA Vaccines

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CONTENTS

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1. INTRODUCTION

Among the most important goals for modern immunologists are finding systemic approaches to curing allergic and autoimmune diseases and developing vaccines that stimulate stronger immune responses against pathogenic organisms and cancer. Vaccines composed of genetic material, either DNA or RNA, promise all the benefits of existing vaccines without the risk of infection. Ideal vaccines should be inexpensive, stable, and easy to manufacture and store. In fact, tremendous progress in genomics and biotechnology have given us hope that construction of such “smart” vaccines will be possible in the near future.

DNA itself is not immunogenic, and this is not surprising since the horizontal flow of antigenic genes would be a major evolutionary force (1). Thus, the genome of vertebrates might not be protected against “genomic invaders,” and recent advances in sequencing of the human genome have revealed the existence of numerous “parasitic” DNA sequences, suggesting the possibility of multiple genomic invasions in the past”(2). This implies that foreign DNA in its purest form, commonly referred to as “naked” DNA, may be ignored by the host’s immune system. In order to be seen as an invader, the message encoded in DNA must be translated into protein, thus revealing itself to the

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immune system. In this simple reasoning lies the essence of DNA vaccination. DNA by itself has a very conserved structure that is almost invisible to the immune system. However, after delivery of DNA to an organism, the encoded message is translated into specific proteins, which can be processed and presented by professional antigen-presenting cells (APCs) providing the initiation of an antigen-specific immune response.

Though Edward Jenner is considered the father of vaccination, there is a controversy regarding the origin of DNA vaccination. Almost all successful endeavors have many fathers, and so does DNA vaccination. Many authors cite the landmark publication by Wolff et al. (3), published in 1990, demonstrating that direct intramuscular inoculation of plasmid DNA encoding several reporter genes led to their expression within the muscle cells. This important contribution demonstrated that *in vivo* administration of “naked DNA” was possible without carriers, such as liposomes (4), or polylysine-glycoprotein complexes (5). This study is predicated by the work of Szybalska and Szybalski, who pioneered the calcium-phosphate method of mammalian cell transfection with exogenous DNA (6). It took the next 30 yr before the potential of protein expression after *in vivo* delivery of DNA for immunization gained wide attention. In 1992 Tang et al. (7) demonstrated that mice injected with plasmid DNA encoding human growth hormone (HGH) could elicit antigen-specific antibody responses. A year later Ulmer et al. (8) published a study showing efficient vaccination of mice with DNA encoding hemagglutinin (HA). High titers of hemagglutinin-inhibiting (HI) antibodies, generation of cytotoxic T-lymphocytes (CTLs), and efficient immunity in “naked” DNA-vaccinated mice opened a new era in DNA vaccination. It is just a matter of time before “vaccinogenomics” will become a new discipline.

2. HELPFUL HINTS

Gene therapy, the strategy of replacing a defective gene(s) to treat a disease, developed rapidly based on the promise of the potential for selective tissue targeting. In its simplest form, gene therapy implies delivering a gene directly into the improperly functioning cell(s) and replacing a malfunctioning gene product without side effects, such as stimulation of the immune response. Moreover, in some circumstances it would be ideal if a gene, once introduced into a cell, could stay active as long as required, possibly throughout the entire life of an individual. This is in contrast to the idea of DNA vaccination, which unlike gene therapy aims at the strongest possible immune response, focused precisely on a specific target, such as a pathogen or tumor-associated antigens, through transient expression of genes encoding antigens and/or immunostimulatory proteins.

DNA vaccines consist of a plasmid DNA backbone containing one or more genes that encode immunogenic and/or immunostimulatory proteins. Once expressed in host cells, these proteins can induce or enhance immune responses. To date this approach has been experimentally applied to elicit protective immunity as well as to induce therapeutically efficient immunity in several species including humans. Both gene therapy and DNA vaccination share similar problems, such as limited therapeutic effectiveness resulting from low efficiency of transfection and lack of specific targeting.

In this chapter we will focus mostly on the therapeutic aspects of DNA vaccines with respect to tumor immunotherapy. We will address some of the current problems and point at possible solutions. Although far from reality, the perfect DNA vector would have certain attributes that could be predicted based on previous experience. These attributes

include: (a) high transfection efficiency of specific cells, (b) maintenance of the episomal state, (c) levels of transgene expression corresponding to expected function, (d) proper intra- or extracellular localization of the transgenic product, (e) stimulation of the proper antigen-specific immune response that is typically humoral for extracellular pathogens and cellular for intracellular parasites and tumor cells.

For clarity, the chapter is organized in the form of a journey starting from the “naked” DNA vaccine entering the organism and following its way through several stages, focusing on the description of major hurdles that need to be overcome to end up with successful therapeutic vaccination (Fig. 1). Finally, we will discuss some of the issues related to the safety concerns over DNA vaccines and the possible future directions of DNA vaccine research.

3. METHODS OF DNA VACCINE DELIVERY AND TRANSFECTION IN VIVO

3.1. *Naked DNA*

The main method of plasmid DNA delivery is the use of needle injection to the muscle or different layers of skin, using saline as a vehicle. Although it is known that naked plasmid DNA transfets nondividing cells very poorly, this inefficient method of transfection requiring large amounts of DNA (usually from 50 µg in mice to 2 mg per dose in humans) is commonly used for its simplicity and the relative efficiency in stimulating immunity. The mechanisms by which plasmid DNA migrates from the extracellular space to the nucleus remains unclear, but because myocytes are multinucleated and their nuclei are in close apposition to the cell membrane it might facilitate nuclear localization of injected plasmids (9). Transcription of delivered transgene(s) in many nuclei at the same time might provide enough mRNA, and consequently protein, falsely implicating that the transgene is being expressed exclusively by myocytes (10). Unlike muscle cells, where the uptake of fluorescence-labeled plasmid DNA is very rapid, the uptake of DNA by monocytes is much slower and detection of mRNA more difficult (10). However, with the use of sensitive RT-PCR techniques, Akbari et al. clearly demonstrated the presence of transgenic mRNA in dendritic cells (DCs) isolated from draining lymph nodes (11). Furthermore, although myocytes seem to be major reservoirs of plasmid DNA in these studies, it is very likely that the lack of costimulatory molecules expressed by these cells limits their role in priming T-cell responses. Since vaccination with plasmid DNA can elicit not only humoral but also cellular immune responses, there must be an efficient mechanism for CTL priming after immunization. In fact there are at least two possibilities: (a) direct transfection of professional APCs and (b) cross-priming in which cells expressing transgene(s) are phagocytosed by APCs and consequently intracellular antigen is processed and presented by professional APCs to T cells. These mechanisms may not be mutually exclusive and might be responsible for significantly better efficiency of gene transfer in regenerating muscle than in normal mature muscle fibers. Regenerating myocytes produce about 80-fold more transgene-encoded proteins than that produced by normal muscle following injection of a plasmid, probably due to more efficient transfection of dividing cells (12). The antigen overexpression might facilitate its uptake by local APCs. Moreover, increased influx of inflammatory cells, which are able to clear up apoptotic cells in regenerating tissue, might be responsible for efficient cross-priming of

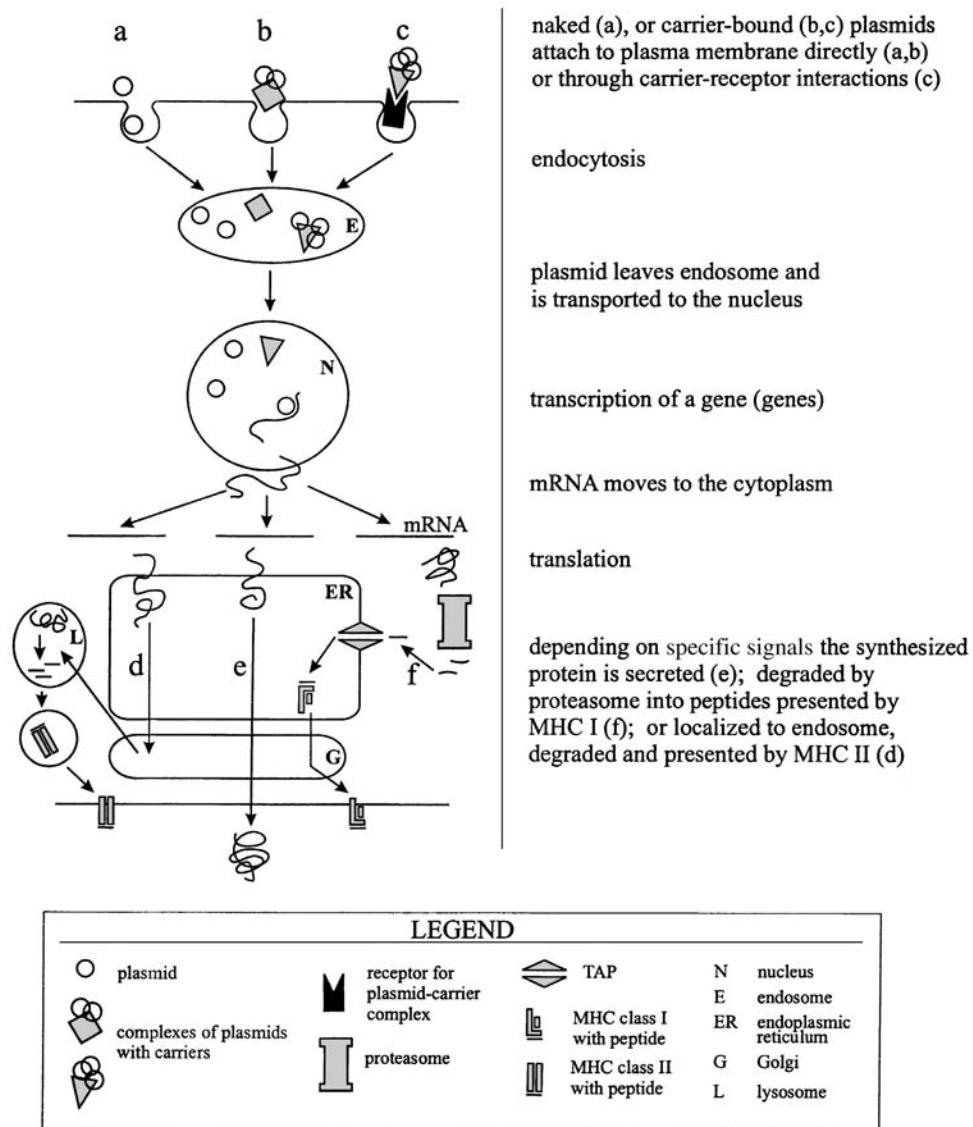


Fig. 1. Schematic representation of the sequence of intracellular events after the administration of a DNA vaccine.

CTLs (13,14). Taken together these data suggest that muscle cells represent a storage and production site for transgene-expressed antigen that is released slowly over a long period of time supporting the persistence of long-term T-cell responses. Several studies indicated the necessity of DC migration between the site of plasmid DNA injection and draining lymph nodes where actual CTL priming is more likely to take place adding a further layer of complexity to the putative mechanism of DNA vaccine-induced T-cell immunity (15,16).

Several techniques based on mechanical, electrical, and chemical approaches have been developed to increase the efficiency of plasmid DNA delivery. Electroporation of plasmid DNA into variety of cells *in vitro* (17), has been successfully adopted for the transformation of cells *in vivo* (18). Although this is an efficient method for site-localized transfection, severe cytotoxicity caused by high-voltage exposure limits its applicability. As shown by Rizutto et al. (19), a significant improvement can be achieved by using low-voltage, high-frequency electric pulses that can increase efficiency of muscle cells transformation 100-fold with only transient tissue damage.

The most effective physical method, thus far, of plasmid delivery is by injection through a “gene gun.” The gene gun uses a regulated burst of helium gas to propel plasmid DNA-coated gold beads (~1–3 µm in diameter) directly into the cytoplasm of skin cells. This method of vaccination typically requires as little as 0.5–2 µg of plasmid DNA to evoke substantial immune responses (20), and is much more reproducible than the syringe injection or electroporation methods (21).

Low-volume jet injection is another mechanical method of plasmid DNA delivery (22). This requires a simple handheld injector to deliver plasmid DNA under high pressure into the desired tissue. It is more effective than syringe injection and without the requirement for precipitation of plasmid DNA onto gold particles, and is simpler to perform than particle bombardment (23). Recently ultrasound was used to increase transfection efficiency of vascular smooth-muscle cells and endothelial cells with wild-type p53 plasmid to prevent restenosis after angioplasty (24). Although the actual blood vessel wall is not used for vaccination, this example shows that there might be other physical methods apart from electric current or the sheer power of gene gun delivery to improve *in vivo* transfection efficiency. Magnetofection based on delivery of DNA-coated superparamagnetic nanoparticles by application of a magnetic field is another interesting experimental approach with efficiency yet to be validated (25).

Obviously using naked plasmid DNA, although sufficient to elicit immune responses, does not always establish a sufficient level of cell targeting. There is an ongoing race between biotechnology companies trying to manufacture a product that would not only improve efficiency of plasmid delivery but also deliver plasmid DNA to the preferred tissue. These efforts are focused largely on the development of nonviral carriers of DNA and are extensively reviewed elsewhere (26).

3.2. DNA Carriers

Organic microparticles, preferably positively charged to increase adsorption of negatively charged DNA molecules, is one obvious choice. Such particles at the size of approx 1 µm were made from poly(lactide-*co*-glycolide) (PLG), which is a biodegradable and biocompatible polymer (27). These particles coated with the cationic surfactants (e.g., 1,2-dioleoyl-1,3-trimethylammoniopropane, DOTAP) proved to be very efficient carriers of plasmid DNA. Moreover, particle-bound DNA induced at least 100 times higher antibody titers than soluble plasmid DNA (27). Similarly, DNA-coated cetyltrimethylammonium bromide (CTAB)/PLG proved to be superior to naked plasmid DNA or even lipofectamine/DNA-transfected DCs in the stimulation of interleukin-2 (IL-2) production by gag55-specific T-cell hybridomas (28).

Other compounds used to improve transfection through compacting DNA are cationic polymers such as poly-L-lysine. This approach offers an interesting possibility for the

formation of cationic polymers substituted with sugar residues as ligands, thus adding a targeting component to the complex. Along this line, plasmid DNA complexed with poly-L-lysine bearing lactose residues was found to efficiently transfect hepatocytes, probably through galactose-specific lectin heavily expressed by these cells (29). Similarly, DNA in complexes with β -D-GlcNAc-substituted poly-L-lysine was efficiently incorporated by cystic fibrosis (CF) epithelial cells (30). However, the use of chemical carriers does not solve all the problems concerning delivery of naked DNA, such as endosomal escape and inefficient nuclear localization.

3.3. Endosome Escape Sequences

The acidic environment of endosomes is detrimental to DNA, thus it is imperative for the plasmid to exit the endosome as quickly as possible. Unlike several viral peptides that facilitate escape of the virus from the endosome, such sequences are probably missing in most plasmids. Apart from providing a component of cellular targeting, cationic polymers can also protect naked DNA from degradation in the endosome. This might be another reason why transfection with cationic lipid-plasmid DNA complexes, which disrupt endosomal integrity, is more efficient than naked DNA (31,32). As much as low pH is destructive to DNA inside endosomes, cytoplasmic DNase poses another serious threat to naked DNA. This DNase activity, which has been shown to be particularly important for the clearance of bacterial DNA in maturing human monocytes (33), might also be destructive for DNA vaccines. Although little is known about this form of DNase in DCs, it might be an important limiting factor in transfection of APCs with plasmid DNA.

3.4. Nuclear Localization of Plasmid DNA

Once released from endosomes into the cytoplasm, plasmids are facing another barrier: the nuclear membrane. Generally, higher levels of transgene expression are observed in actively dividing cells where the nuclear membrane disappears during mitosis (34). However, unless tumor or bone marrow cells are being targeted for transfection most cells, including muscle and dendritic cells, are nondividing. To overcome this barrier plasmids should contain specific nuclear localization sequences. The major feature of such sequences is the presence of the transcription factor-binding consensus sequences. Transcription factors present in the cytoplasm bind to the plasmid DNA and facilitate nuclear entry. A typical example is the 72-bp repeat found in the SV40 enhancer, which possesses several consensus sequences able to bind common transcription factors, such as AP1, AP2, AP3, Oct-1, and NF- κ B (35). Among them AP2 seems to play a crucial role in nuclear localization of the nucleoprotein (35).

An appropriate nuclear localization signal (NLS) can also be provided through linking oligopeptides containing NLSs to the plasmid DNA (36). It appears that linking a single NLS to a given plasmid results in 10- to 30-fold increased transgene expression in non-dividing cells, such as macrophages (37), or 100-fold increase in primary neurons (38). In this case the mechanism of nuclear translocation relies on the interaction of basic amino acids present in the NLS with cytoplasmic factors called karyopherins (39). The complex is docked to the nuclear pore complexes (NPCs) and then translocated to the nucleus (40). The presence of NLSs in the cationic peptide (CL22) used for the plasmid DNA delivery to DCs might be partially responsible for the relatively high (15%) trans-

fection efficiency of DCs (41). Recently substantial progress has been made in the design of short synthetic peptides suitable for the efficient delivery of plasmids (42), and once their immunological properties are known they have the potential of becoming a leading DNA vaccine carrier.

Another exciting way of directing plasmid DNA to the nucleus relies on the complex formation with fibroblast growth factor-2 (FGF-2) that plays a major role in wound healing (43). It has been recently demonstrated that FGF-2 may serve as a fairly efficient DNA carrier (44). Although the mechanism of FGF-2 translocation to the nucleus remains elusive, it has been shown that FGF-2 can act as a direct transcription factor (45). Plasmid DNA coupled to FGF-2 binds to FGFR and the whole complex travels to the nucleus (44). Whether FGF-2 plays a role in previously mentioned increased plasmid uptake by regenerating muscle remains to be established. Nevertheless, there is great potential in using FGF-2 and similar growth factors for tumor-targeted DNA vaccine delivery.

The increased efficiency of nuclear delivery by formation of highly sophisticated DNA-carrier complexes is balanced by the limitation in unpackaging the plasmid DNA cargo following localization to the nucleus (46). It appears that the stability of DNA complexes requires very fine-tuning with intermediate stability being required for maximal gene expression.

Although the above modifications of plasmid DNA are important for increasing the yield of transgene expression, they might prove impractical from an economical point of view. Among the key features of DNA vaccines are its simplicity and the low cost of manufacturing compared to other vaccines. Fortunately, there is a lot of room for improvement of DNA vaccines through the modification of the vector itself, by the use of custom-designed promoters, and by incorporation of immunostimulatory sequences.

4. CHOICE OF PROMOTER

After being successfully delivered to the nucleus the message contained in the transgene needs to be efficiently transcribed to mRNA. A promoter with a tandem of regulatory sequences is responsible for the initiation of transcription and subsequently for the level of protein synthesis (for review, see 47). Unless there is a major breakthrough in the development of more efficient methods of plasmid DNA delivery, we must rely on the use of strong and, unfortunately, tissue nonspecific promoters. The most commonly used in DNA vaccines are viral promoters, such as the human cytomegalovirus (CMV) immediate-early promoter (IE-CMV), the Rous sarcoma virus (RSV) long terminal repeat (LTR-RSV), and the simian virus 40 (SV40) promoter. Among them by far the most popular is the IE-CMV promoter, often used with an adjacent enhancer (48). However, the activity of this promoter is detected in only a handful of tissues including muscle and skin (49), and is strongly dependent on their developmental state (50). Moreover, the promoter has a tendency for shutting down in vivo, that might be related to the suppressor activity of p53, thus raising a cautious note for targeting nontumor cells with constructs containing this promoter (51). In monocytes the activity of IE-CMV promoter is strongly influenced by mobilization of NF- κ B by tumor necrosis factor- α (TNF- α) (52). It appears that other transcription factors, such as C/EBP- α and - β , involved in maturation of monocytes, can suppress NF- κ B-mediated upregulation of the IE-CMV promoter (53). This pattern of IE-CMV promoter regulation may have major consequences for the expression of transgene(s) delivered by plasmid DNA to APCs. Such a mechanism may

not only facilitate IE-CMV promoter–controlled transgene expression in directly transfected immature dendritic cells (IDCs) but may also ensure its transient expression in mature DCs. This might help to explain the relatively high efficiency after delivery of plasmid DNA containing IE-CMV promoter by intramuscular or intradermal injection. Thus, the efficiency of the IE-CMV promoter in eliciting an immune response may be based on the high level of expression in transfected cells or may be a result of the selective expression in specific tissues.

Properly targeted APCs may be much more efficient in priming immune responses without the risk of generating autoimmunity. This might be achieved by using tissue/cell-specific promoters that could be found in sequences of genes whose expression is specifically upregulated in DCs. One of them is B7.1/CD80, a potent costimulatory molecule upregulated during DC maturation (54). Regulatory elements contained inside 3084 bp of the 5'-sequence of the putative B7.1 promoter have been shown to be sufficient to provide high expression of a reporter gene in APC-rich tissues (55,56). However, although such inducible promoters should be advantageous over constitutive promoters, they are difficult to handle largely because of plasmid size constraints. To maintain tissue specificity the presence of many upstream regulatory elements are required, and if truncated to fit the right size of the plasmid, such promoters often lose their strength and specificity (57,58). Nevertheless, an inducible expression system based on the combination of hypoxia-responsive element (HRE) and hypoxia inducible factor-1 (HIF-1) has been shown to be effective for driving transgene expression in usually hypoxic solid tumor tissue (59). This system might be particularly useful for the introduction of major histocompatibility complex (MHC) class I/II or costimulatory molecules into the tumor cells to enable efficient presentation of tumor associated antigens (TAAs). However, for the purpose of DNA vaccination this expression system would require further strengthening. One of the approaches to this problem might be in designing chimeric or an artificial inducible promoter combining both strength and specificity. Such expression systems, allowing control of the level and timing of gene expression, were constructed based on the combination of pharmacologically regulated hybrid transactivators with bacterial repressors (60,61). One contains the *Gal4* DNA-binding domain from yeast fused to the activation domain of the NF-κB p65 to produce a very powerful transactivator. Even relatively low expression of such transactivators induced by radiation, heat shock, or pharmacological agents proved to be sufficient for decent expression of the reporter genes (62). Moreover, a similar system of transactivation was also successfully applied to HRE/HIF-1 regulated expression (63). Recently Stanojevic et al. (64) demonstrated construction of a completely artificial transactivator, thus raising new hope that novel, nonviral promoters can be used in DNA vaccines. If this work is confirmed, such transactivators placed under the control of a weak, but tissue-specific promoter, could efficiently amplify expression of either cis- or transpositioned genes regulated by transactivator-controlled promoters. This concept of “promoter cascades” has been shown to be efficient in the expression systems based on *fhp/FRT* and *cre/LOX* recombinases (65,66). However, potential immunogenicity of the regulatory elements utilized in these systems might pose a major drawback for in vivo application of these highly sophisticated “artificial” expression systems.

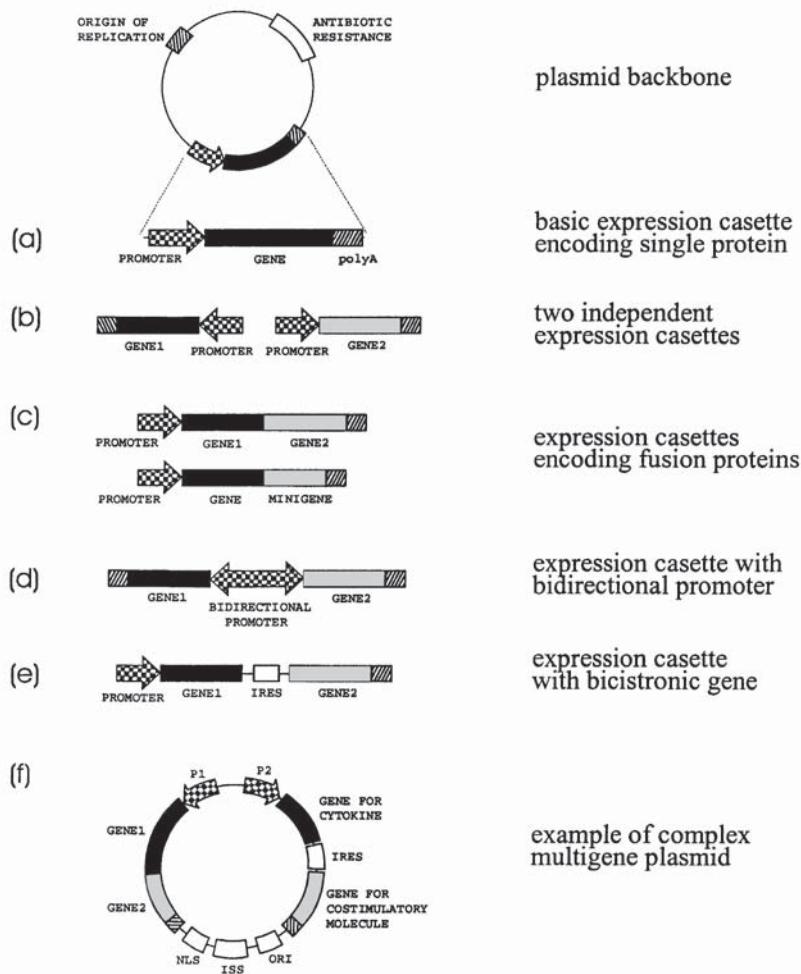


Fig. 2. Examples of plasmid constructs designed for DNA vaccines.

5. PROCESSING OF TRANSGENE-ENCODED PROTEINS

The DNA vaccine in its simplest form contains a single gene with an open reading frame (ORF) for a single protein (Fig. 2). Importantly, protein sequence, structure, and processing have a major impact on the future quality of the immune response. The fate of a protein depends on the presence of the localization signals (LSs) it contains. Lack of any LS results in intracytoplasmic retention of the protein and significantly increases the chances of presentation by MHC class I molecules following proteasomal cleavage (67). The efficiency of processing through the MHC pathway may be further increased by incorporation of ubiquitination signals into the protein. According to the so called “N-end” rule, the presence of significant hydrophobicity at the N-terminus of a polypeptide chain can facilitate conjugation of ubiquitin to the protein, thus increasing its pro-

teolysis (68), and the subsequent presentation of immunogenic peptides bound to MHC molecules (69–71). This mechanism might be particularly important for upregulating presentation of an antigen by directly transfected professional APCs and skewing the immune response toward a Th1 phenotype (72). Though being important for the generation of epitopes from long polypeptide chains, ubiquitination might be fatal for the vaccine containing minimal epitopes that are already preprocessed to fit the MHC class I-binding surface. In this case incorporation of an endoplasmic reticulum (ER)-targeting signal can circumvent proteasomal degradation by translocating the peptide into the ER, where it can be efficiently loaded onto MHC molecules (73). A similar approach was used to target proteins into the MHC class II-processing pathway involving endosomal and lysosomal compartments. The lysosome-targeting signal containing tyrosine-based sorting motif of lysosomal membrane protein (LAMP-1) was added to the antigen (74), resulting in significantly improved presentation of antigen to CD4+ T cells (75,76).

Immunization with soluble protein or extracellular localization of transgene-encoded protein is a standard method for generating a humoral response, which is regulated by Th2 CD4+ T cells (77). To ensure extracellular localization of transgene-encoded proteins, inserted genes have to contain a sequence coding for a signal peptide, whereas all sequences coding for potential membrane-anchoring signals, transmembrane domains, and specific retention signals (if present) need to be removed. To increase transport of the protein out of the cell a native signal sequence might be replaced with optimized artificial sequences (78). This maneuver should be very efficient for the generation of humoral responses after administration of DNA vaccines. On the other hand, inclusion of the anchoring signal in transgenic proteins expressed in tumor cells may not only improve their recognition as target cells but may additionally spur cross-presentation of the antigen (79).

6. ORGANIZATION AND CONTENT OF DNA VACCINES

6.1. *Multigene Plasmids*

Theoretically, administration of multiple plasmids containing genes for cooperating proteins is possible and easy to perform. However, considering the low efficiency of transfection, the chance of being colocalized to ensure optimal cooperation is very small. Thus, innovative methods for incorporating more than one therapeutic gene into a single plasmid became essential for optimizing DNA vaccines. Several approaches to solve this problem have been proposed. The simplest method is cloning of an additional expression cassette (promoter-gene-polyA) into the vector, although caution has to be exercised to avoid cross-promoter silencing (*see* Fig. 2B; M. Sektas, personal communication). Along this line bidirectional promoters have been used allowing the unilateral expression of two transgenic proteins: HbsAg and HbcAg (80; *see* Fig. 2D).

Despite the ongoing controversy over the existence and nature of the internal ribosome entry site (IRES) in eukaryotes (81,82), some mRNAs in eukaryotes can be multicistronic, thus allowing production of several proteins from a single transcript (Fig. 2E; 83). Moreover, IRES-mediated translation, although relatively weaker than the conventional cap-dependent translation, has been shown to be active in cells exposed to extremely stressful conditions such as gamma-irradiation, hypoxia, or amino acid starvation (81). This makes IRES-controlled initiation of translation a prime choice for the intratumoral expression of transgenes. Such plasmids coding for bi-cistronic mRNA of human IL-5

and IL-1 β have been constructed and were functional (84), but the real power of this approach was demonstrated in the combination of HIV-1 gp120 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (85). Immunization of mice with a bi-cistronic plasmid that coexpressed gp120 and GM-CSF under control of a single promoter, was superior to an admixture of plasmids expressing GM-CSF and gp120 DNA vaccine in stimulating a gp120-specific CD4+ T-cell response. However, one has to keep in mind that the addition of a potential immunostimulator will not always augment immunity. For example, the combination of HIV-1 gp120 and IL-2 in a bi-cistronic vector might induce weaker specific immune responses than the mono-cistronic plasmid coding for gp120 alone (86). This is not necessarily surprising since gp120 is a powerful immunogen and priming in the presence of IL-2 may lead to depletion of responding T cells secondary to activation-induced cell death.

6.2. Plasmids With Genes for Fusion Proteins

Another approach to multifunctional DNA vaccines is based on covalent linking of two or more proteins (Fig. 2C). A number of DNA vaccines containing genes encoding fusion proteins have been generated. The array of possible applications of such vaccines ranges from inhibition of allergic reactions (87), through suppression of autoimmunity (88,89), to boosting vaccine-induced immunity (90). Here we present only a few out of many promising applications of this technology.

The access of antigens to APCs appears to be a rate-limiting step in the generation of immune responses by DNA vaccines. Fusion of genes encoding an antigen with a common ligand of receptors present on the surface of APCs may enhance the processing and presentation of the antigen. For example, Drew et al. reported enhanced responses to DNA vaccines encoding a fusion protein composed of an antigen and the cytotoxic T lymphocyte antigen-4 (CTLA-4) that binds with high affinity to the B7 membrane receptor on APCs (91). This novel approach serves two purposes at the same time: it facilitates uptake of antigen by APCs, and also blocks interactions that provide inhibitory signals to activated T lymphocytes through CTLA-4 present on their surface (92,93).

Similarly, other costimulatory molecules, such as CD40L, have been fused to selected tumor antigens in an effort to improve immunogenicity (94). In this case CD40-CD40L interaction improves antigen presentation and consequently enhances priming of T cells owing to CD40L-stimulated maturation of APCs (95). Inclusion of sequences coding for CTLA-4-antigen or CD40L-antigen into the DNA vaccine not only can improve processing of the antigen but also facilitates its proper targeting to secondary lymphoid organs.

To date, one of the most popular approaches for improving the immunogenicity of DNA vaccines has been the use of fusions of different immunomodulatory cytokines to the Fc portion of immunoglobulins (Igs). The major advantage of using Fc-cytokine fusion proteins is their prolonged half-life and the ability to cluster receptors owing to dimerization of the Fc segment. In this model of fusion proteins Fc is mutated to eliminate antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-binding properties (86). On the other hand, fusion of an antigen to the intact Fc portion of IgG facilitates internalization of the Fc-antigen complexes and promotes efficient MHC class I- and II-restricted antigen presentation (96,97). Interestingly, not only Fc but also Ig variable regions (sFv) have been used to improve immunogenicity of proteins. Lymphoma sFv were fused respectively to two chemokines, interferon inducible protein-10 (IP-10) and monocyte chemotactic protein-3 (MCP-3). The sFv-chemokine fusion proteins elicit-

ited chemotactic responses in vitro and induced inflammatory responses in vivo. These fusion proteins showed superior chemoattractive properties and subsequent tumor protection as compared to the chemokine alone (98).

Cytokine-antigen fusion proteins have been shown to be especially effective in stimulating cellular immunity. Among several ovalbumin (OVA)-cytokine fusion proteins, those containing GM-CSF, IL-10, and IL-12 proved to be the most efficient in an OVA-expressing murine tumor model. Interestingly, a nonapeptide from IL-1 β (163–171) fused to OVA was as potent as IL-12 in inducing a Th1-type response (99). This approach might be especially interesting for tumor therapy with TAA-cytokine fusion proteins.

Linkage of antigens to heat shock proteins (HSPs), which play a role as chaperones involved in the endogenous antigen-processing pathway, represents a particularly interesting approach for increasing the potency of DNA vaccines. Several members of the HSP70 and HSP90 family have been shown to facilitate CTL priming when injected in the form of protein-based vaccines (100). Incorporation of the gene encoding HSP70-antigen fusion proteins into DNA vaccines was effective in enhancing CTL priming, followed by potent antitumor immunity against established tumors (101). Similarly, fusion of the antigen to N-terminal HSP-binding domain of the SV40 T-large antigen to increase chaperoning by endogenous HSPs, led to increased activity of antigen-specific CTLs (102).

Finally, the approach of using fusions of different coding sequences to improve potency of DNA vaccines was applied to minigene-encoded T-cell epitopes. It appeared that relatively low-efficiency T-cell induction by minigene DNA vaccines could be enhanced several-fold by fusing minigenes to C-terminal coding sequences of the carrier protein (103).

6.3. Minigenes-Based Polyepitope Vaccines

Revealing the molecular mechanism of MHC/peptide recognition by $\alpha\beta$ T-cell receptors (TCRs) (104) allowed the identification of a large number of immunogenic epitopes (105). Synthetic peptide combinatorial libraries proved to be a powerful tool for screening millions of peptide candidates, thus greatly speeding up discovery of new immunogenic epitopes (106), with immediate consequences for the design of vaccines. Numerous immunogenic epitopes have now been identified in many potential targets of vaccination ranging from important clinical pathogens, such as HIV, to antigens from a wide variety of tumors. Instead of using the entire coding region of an antigen in plasmid DNA, only a single epitope or several epitopes with known immunogenicity can be sequentially arranged into a single polypeptide, thus forming a string of epitopes. This so-called minigene or minimal-epitope approach takes advantage of the fact that plasmid DNA-encoded antigenic peptides can be loaded onto MHC class I molecules via the endogenous antigen-processing pathway. One of many examples of polyepitope vaccines that recently entered clinical trials is the polyepitope HIV-1 vaccine that is currently being evaluated in prime-boost regimens in Kenya (107). Several precautions have been taken into consideration while constructing this vaccine including: (a) fusion of coding sequence of 25 partially overlapping CTL epitopes to C-terminal coding sequence of truncated gag, (b) prevention of membrane localization of the recombinant protein, (c) inclusion of MHC class II-restricted epitopes, (d) inclusion of a 12-bp-long Kozak consensus sequence to optimize translation, (e) codon usage optimized for human genes, and (f) choice of multiallelic epitopes to cover a broad range of HLA alleles. The safety and

efficiency of this vaccine was confirmed in several preclinical studies (108,109). It will take several years before the actual efficacy of this vaccine is known, but in the meantime, new improvements will certainly be introduced based on the growing knowledge of interactions between epitopes. For example, it was recently shown that certain HIV-1 epitopes have the potential to antagonize immunogenicity of others through the formation of junctional epitopes (110). Once such obstacles are eliminated, polyepitope DNA vaccines might prove superior to vaccines relying on the entire coding region of an antigen.

6.4. Immunostimulatory Sequences

Immunostimulatory sequence (ISS) DNA, also known as CpG motifs, is a family of unmethylated CpG dinucleotides with a consensus sequence of 5'-pur-(pur or T)-CpG-pyr-pyr-3"(111). These sequences, which are common in certain bacterial and viral genomes but suppressed in mammalian genomes, have a broad range of stimulatory effects on natural killer (NK) cells, B cells, and APCs. Furthermore, cytosine methylation in CpG dinucleotides has also been recognized as an important element of transcriptional regulation in vertebrates. DNA methylation has been postulated to play a major role in the inactivation of transcription of foreign genes, transposons, and retroviruses (112). A recent study showed that addition of CpG motifs adjacent to an RSV promoter could prevent its transcriptional suppression (113). Although little is known about mechanisms silencing promoters in episomal DNA, it is possible that CpG methylation might be involved in promoter regulation. It is tempting to speculate that proper positioning of CpG motifs in DNA vaccines might have a beneficial effect on their efficacy not only through an ISS effect but also through guarding promoter activity.

7. MECHANISMS OF DNA VACCINE-INDUCED IMMUNITY

The ability to “sneak in” to an organism without inciting a strong immune response makes plasmid DNA unique among vaccines. In fact, complete adaptive immunity specific to plasmid DNA has not been reported. Importantly, although plasmid DNA is quickly deposited in myocytes and APCs (10), there is a several-hour time gap between injection of DNA vaccine and the appearance of encoded antigen. This might have major consequences for the ability of DNA vaccines to prime naïve T cells. The amount of antigen produced in vivo after DNA inoculation is in the picogram to nanogram range (3). Equivalent amount of antigen introduced in vivo in the form of protein would probably be processed by macrophages without inducing an adaptive response, resulting in the impression that the antigen was either ignored or tolerized. These observations suggest that the plasmid itself may function as a powerful immunomodulator (Fig. 3).

Receptor-mediated activation of monocytes is the most likely mechanism of DNA-induced immunomodulation. Among numerous Toll-like receptors (TLRs) that function as pattern recognition receptors (PRRs) to initiate the innate immune response (114), only TLR-9 expressed on the surface of B cells, DCs, and NK cells has been shown to recognize ISSs, particularly unmethylated CpG motifs in bacterial DNA (115,116). Incorporation of a cassette containing repetitive GACGTT sequences for vaccines tested on mice, or GTCTGTT in humans, may significantly boost its potency and skew the immune response toward a Th1 phenotype. The role of CpG motifs in stimulating immunity was recently reviewed (111,117,118).

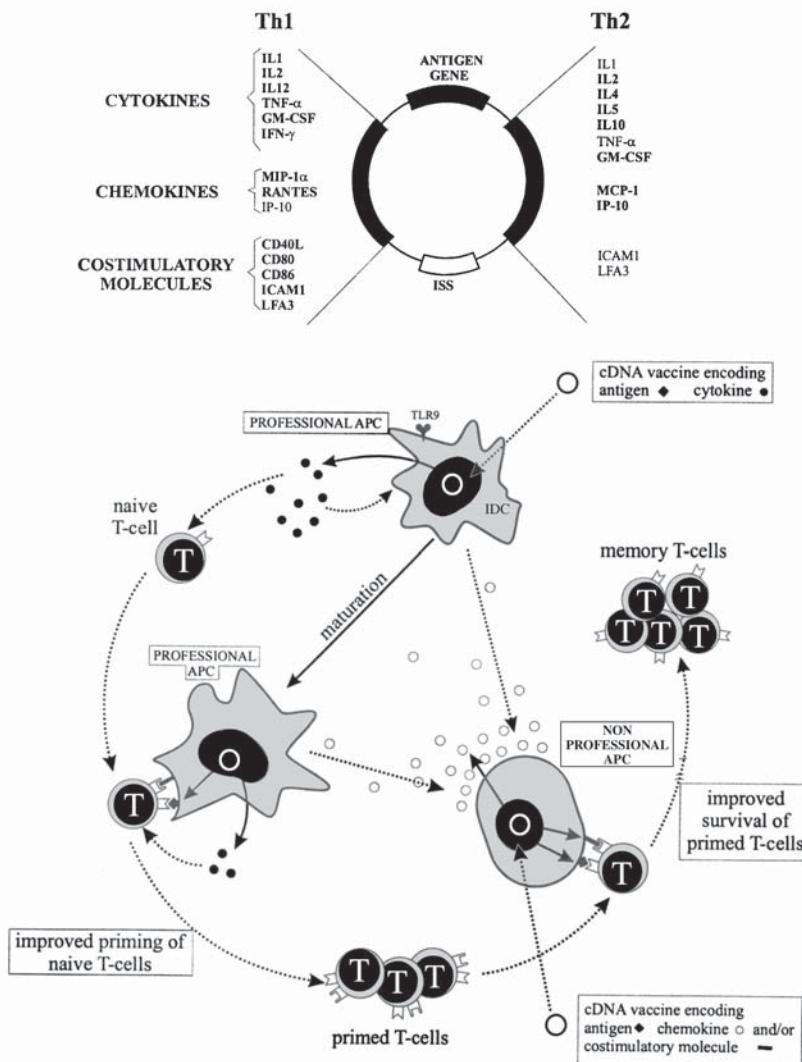


Fig. 3. (A) A model DNA vaccine encoding antigen and cytokines, chemokines, and/or costimulatory molecules, promoting Th1- or Th2-type immune responses. The coexpression of specific immune molecules can influence the type of immune response against individual antigens. **(B)** Schematic representation of the induction of T-cell immune responses following administration of a DNA vaccine. Injection of plasmid DNA leads to transfection of professional (DC) and nonprofessional APCs (myocytes, epithelial, and endothelial cells). Following transfection, immature DCs (IDCs) become mature DCs (MDCs) and are able to prime T-cell responses. The survival and expansion of antigen-specific T cells is maintained by interactions with plasmid-transfected nonprofessional APCs.

Activation of signaling pathways in DCs leads to the production of not only IL-12, important for skewing immune responses toward Th1(119), but also TNF- α and Type 1 interferons that are important for the release of IFN- γ by NK cells (120). Type 1 interferons appear to be particularly important for the stimulation of Th1-type priming by ISS-containing DNA vaccines. It has been shown that IFN- α and IFN- β secreted by ISS-stimulated APCs can upregulate not only B7 but also peptide transporter associated with antigen processing (TAP) expression (121), which is essential for efficient antigen cross-presentation (14). Moreover, recently published reports indicate the importance of IL-18 and IL-23 in the Th1-polarizing capacity of DCs (122,123). Altogether, these observations might explain why gene gun delivery of plasmid DNA produces predominantly Th2-type response. Simply, gene gun forces a large portion of DNA vaccine straight into the cytoplasm, thus omitting receptors involved in DNA-induced immunostimulation. It appears that even supplying DNA vaccines with additional CpG motifs that stimulate Th1 responses cannot overrule Th2-promoting signals delivered by gene gun bombardment (124).

It becomes clear that long before plasmid-encoded protein is even expressed there is already an ongoing innate immune response able to amplify processing and presentation of the antigen by APCs. Successful immunization requires all steps of DC maturation from monocyte through IDC to MDC to occur, with each cell playing a critical role in DNA-elicited immunity (125,126). The early presence of IFN- γ that is essential in stimulating maturation of monocytes and upregulation of several costimulatory and antigen-presenting molecules, such as MHC class II, B7.1/CD80, B7.2/CD86, ICAM-1/CD54, and CD40 seems to be particularly important (127). IFN- γ can also significantly influence the intracellular processing of antigen by inducing replacement of standard proteasomes by immunoproteasomes that are much more efficient at producing antigenic peptides presented by MHC class I to CD8+ T cells (128,129), thus explaining the efficient priming of naïve CD8+ T cells by DNA vaccination (130). This two-phase response allowing DCs to reach a more mature phenotype characterized by the expression of several costimulatory molecules at the time of antigen presentation may also have beneficial effects through lowering the threshold of MHC/peptide-TCR binding avidity. This way more precursors including those with relatively low-affinity TCR, have a chance to be activated, thus increasing the polyclonality of T-cell responses.

The magnitude of immune induction depends upon frequency of encounters between naïve T lymphocytes and APCs within lymph nodes. Migration of directly transfected DCs to secondary lymphoid organs is critical for priming immune responses following DNA vaccination (11,15). Although the ability of naked DNA to induce migration of DCs is yet to be proved, almost certainly plasmid DNA-microparticle complexes can be phagocytosed by monocytes at the site of injection followed by migration to lymph nodes where they become perfectly functional DCs (131). DNA vaccines can be improved by including genes encoding chemokines involved in the migration of DCs. Receptors for chemokines such as CXCR4, CCR4, and especially CCR7 are upregulated in maturing DCs (132). The CCR7 ligands, secondary lymphoid chemokine (SLC/CCL21) and Epstein-Barr virus-induced molecule-1 ligand chemokine (ELC/CCL19), have been shown to be potent immunostimulators for humoral and T-cell-mediated immunity (133). Overexpression of these chemokines in plasmid-transfected cells may increase chances

of direct contact between T cells and DCs accumulating along an increasing gradient of the chemokine, thus leading to improved presentation of coexpressed antigen.

Once primed, lymphocytes require favorable conditions to survive and expand. For example, Th1-type cells must reencounter antigen soon after differentiation to avoid activation-induced cell death (134). Antigen expressed in transfected muscle fibers can secure sufficient levels of antigen available to sustain Th1-type cells. Additional protection of primed T cells can be provided by properly formulated plasmid DNA. For example, 4-1BBL expressed on APCs generates signals necessary for survival of activated T cells by interaction with 4-1BB (CD137) (135). Coexpression of an antigen and 4-1BBL from the same plasmid in nonprofessional APCs may efficiently prevent deletion of antigen-specific T cells.

Persistence of low levels of antigen in the periphery may be pivotal for maintaining protective immunity. In this sense, the weakness of currently used DNA vaccines that produce small amounts of proteins might represent a beneficial role in supporting survival of memory T cells. More efficient DNA vaccines could generate substantially more antigen, but instead of improving immunization they might cause substantial detriments in the milieu of an ongoing immune response. On the other hand, low doses of presented antigen assure survival of high-avidity T cells that are crucial for an effective CTL response (136,137). This is the basis for prime-boost strategies in which DNA vaccines are followed by viral vectors.

The time lapse between entry of the transgene and expression of transgene-encoded protein results in the “unannounced” appearance of antigen in the secondary lymphoid organs. The appearance of the antigen in lymph nodes can produce sufficiently strong danger signals to initiate priming of T cells and also supports proliferation of existing memory cells leading to a strong CTL response (138,139). In fact, intralymphatic administration of DNA vaccine is 100- to 1000-fold more efficient than immunization via conventional routes (140). This observation fully supports a thesis formulated by Zinkernagel (141,142), that delivery of small amounts of antigen to secondary lymphoid organs and transient expression within the nodes is essential for induction of protective immunity. Inclusion of proper immunomodulators in DNA vaccines delivered into lymph nodes (LNs) might significantly improve this method of immunization without necessarily providing larger amounts of antigen. For example, it would be interesting to create DNA vaccines encoding an antigen and IFN- γ -inducible protein-10 (IP-10), which causes retention of Th1 lymphocytes in draining LNs, thus facilitating their contact with DCs and improving presentation of weak antigens (143). This kind of vaccine formulation might be particularly useful for tumor vaccines since it combines weak TAAs with IP-10, and has already demonstrated an effective tumor-protective CD8+ T-cell response in mice (144). This is just a single example of a multigene DNA vaccine encoding an antigen and immunomodulatory molecule. To date the efficacy of at least 60 different combinations of cytokines, chemokines, and costimulatory molecules were examined in several experimental settings, with most of them being able to improve vaccine-induced immunity (72,118,145). However, evaluating the optimal composition of DNA vaccines for possible use in humans is challenging because of the enormous heterogeneity of individual host molecular and clinical profiles, which may require a more personalized approach.

Current knowledge of the mechanism by which central and effector memory cells are generated is limited (146). In fact, it has been suggested that only those vaccines that

induce long-term protection exert this effect through neutralizing antibodies (147). Apparently, antigen-specific T-cell precursors induced by vaccines aimed at HIV or antigenic tumors do not persist for long enough to maintain sufficient numbers of activated effector T cells. Although the mechanisms of terminating immune responses are incompletely understood, it is possible that eradication of antigen from the host could be a major reason for termination of the immune response (142). Though this explanation might be plausible for certain kinds of transient infections, there is a little chance for complete elimination of an antigen in chronic infections or cancer. Along this line, efficient control of chronic infections might rely on a continued immune response maintained by functional memory B and T cells. Long-lasting expression of proteins provided by DNA vaccination may favor persistence of T-cell memory and consequently effective immunity (3).

8. SAFETY CONCERNS AND ETHICAL ISSUES

As much as gene therapy raises important ethical questions, they may not be directly applicable to DNA vaccination. By questioning DNA vaccines we might start questioning the whole concept of vaccination as well, since in many cases traditional vaccines contain microbial DNA. Routine immunizations have been remarkably successful in inducing resistance to infections with several common pathogens. This, however, may come at a price since some researchers have postulated that because of elimination of certain pathogens, the human population became more vulnerable to infections with newly evolving pathogens (148). If one considers the relationship between the evolution of the human immune system and the evolution of pathogenic microorganisms as a race for survival (149), there cannot be a winner. The mass-vaccination programs over the last 50 yr have likely shaped biological processes influencing natural selection in the human population. However, withholding vaccination in an effort to reintroduce Darwinian natural selection cannot be considered for many ethical, sociological, and economical reasons (150). This general principle may also apply to the growing epidemic of cancer.

Genomic integration of plasmid DNA is among several concerns connected to the application of DNA vaccines. Although a justifiable concern if considering mass use of gene replacement technology in the population, it may be less important when considering the limited use in terminally ill cancer patients. In essence, cancer patients already carry deregulated genes and the possibility of integrating an additional piece of DNA carrying potential treatment may be less objectionable. Moreover, the probability of plasmid integration to genomic DNA is very low (151,152), and considering the small percentage of coding and regulatory sequences in the human genome (153), the probability of plasmid integration into these functionally important regions is practically negligible. Other concerns, such as the potential for induction of immunologic tolerance or autoimmunity, and possible induction of anti-DNA antibodies, also have very little scientific confirmation at this time (154).

Owing to the rapid development of DNA microarrays and proteomics technology it has become clear that, unlike other epidemic diseases, the treatment of cancer requires an individualized approach. Because of the enormous molecular heterogeneity of cancer and individual variations in the human genome, no single drug or even combination of drugs will be able to eradicate phenotypically similar tumors in all patients (155). The same may apply to other immunotherapeutic methods as well. However, DNA vaccines,

unlike other therapeutics, can be relatively easily customized according to individual needs.

Other issues facing DNA vaccine development are current procedures involved in early-stage research and development (R&D) that often require lengthy procedures of clinical evaluation and complex patenting requirements resulting in excessive costs. This might pose major drawbacks for obtaining new therapeutically valuable vaccines. The assignment of DNA patents allows industry to maintain profitability and continued interest in vaccine production and evaluation. However, the same patent laws monopolize R&D and may also increase the cost of research. Although this will continue to be debated by ethicists and legal scholars, the scientific support for DNA vaccines and the low cost of production will likely push the field forward.

Obviously more studies are required to ensure the safety of plasmid DNA inoculation into humans. However, as long as immunotherapy is considered the last resort after traditional methods of therapy have failed, there is less chance for DNA vaccines to prove their true value. Further research of DNA vaccines and other forms of immunotherapy need to be evaluated in patients with earlier-stage disease and at a time when they have not been weakened by prior exposure to cytotoxic agents.

9. CONCLUSIONS AND FUTURE DIRECTIONS

A few thousand papers have been published on the use of DNA vaccines across several species. Most of them support DNA as an excellent immunizing agent, particularly for priming immune responses. Plasmid DNA used as a part of a therapeutic cancer vaccine strategy certainly has the potential to induce tumor-specific immunity and possibly regression of disease in cancer patients. Challenges for the future will be: (a) to identify more immunogenic and specific antigens, (b) to construct more efficient and tissue-specific promoters, (c) to improve methods of tissue-targeted DNA vaccine delivery, (d) to optimize processing and presentation of transgene-encoded antigens, (e) to design optimal combinations of DNA vaccine content corresponding to the individual needs of a patient. Current progress in the human genome sequencing and rapid advances in genomic expression profiling and functional proteomics are key factors that will allow creation of such “smart” vaccines. Identification of new antigens through analysis of genomic libraries and better knowledge of protein–protein interactions may hasten this goal in the foreseeable future.

Edward Jenner was likely unaware of the full impact of smallpox vaccination, yet his vaccine proved to be powerful and efficient resulting in the eradication of smallpox in modern times. This represents an important lesson in that we might never learn the exact mechanisms governing vaccine-induced immunity and be able to control all possible consequences of using DNA for vaccination. However, if one considers the similarities between smallpox and cancer as major epidemic diseases, the application of effective immunotherapy, including DNA-based vaccines, should be explored to the fullest possible extent.

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1. INTRODUCTION

Although identification of tumor antigens has propelled the development of many different approaches to cancer immunotherapy, whole-tumor vaccines continue to be of interest because they contain a large number of antigens, known and unknown, allowing for both widely applicable as well as patient-specific vaccines. Although the presence of a progressing tumor might suggest that whole-tumor cells would not be immunogenic, mouse experiments performed 60 yr ago first demonstrated that tumor cells could elicit immunity and protect syngeneic mice from a subsequent tumor challenge (1,2). In fact, in one model, cell-associated antigen was associated with better presentation of antigen to CD8 T cells than soluble antigen (3). Thus, it is clear that how the immunizations are performed or how modifications are made to the tumor to increase immunogenicity are important for activation of tumor-abrogating immune responses. For example, tumor cells may be injected along with potent inflammatory adjuvants or they may be genetically altered to express cytokines, human leukocyte antigen (HLA) or costimulatory molecules. This chapter will discuss the scientific basis for whole-cell vaccines, whereas others will discuss cytokine-modified tumor-cell vaccines, haptenated tumors, tumor-cell lysates, or shed tumor cell antigens.

2. IMMUNOLOGIC MECHANISMS

The cross-priming model of activating naïve T cells against tumor antigens proposes that when injected tumor cells die, they or their fragments, are taken up by *in situ* dendritic cells (DCs). These DCs, following migration to regional lymph nodes, process and present

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the tumor antigens to naïve T cells bearing receptors for that antigen along with the necessary costimulatory molecules to activate an antigen-specific immune response. More recently, an additional model for T-cell activation has been proposed by Zinkernagel's group (4). They suggested that tumors can activate cytotoxic T lymphocyte (CTL) responses directly if they reach secondary lymphoid tissue in sufficient numbers and for a long enough period of time following subcutaneous or intrasplenic injection of a tumor-cell suspension. Furthermore, if tumors remained outside secondary lymphatic organs, or are segregated from T cells by physical barriers (such as collagen deposition), they were ignored by T cells. Thus, antitumor immunity generated with whole tumor cells may be achieved equally well via direct-priming that required CD4+ help and cross-priming, which may be independent of CD4 help (5). Others have suggested that different mechanisms may be involved in tumor prophylaxis (CD8-independent) compared to tumor therapy (CD8-dependent) (6).

3. ADJUVANTS FOR TUMOR VACCINES

Regardless of the model for T-cell activation, because tumors express self molecules, to which the immune system may be tolerant, and because the tumor environment may contain immunosuppressive molecules such as interleukin-10 (IL-10) and TGF- β , additional "danger" signals will be needed to properly activate the immune response. The most frequent manipulation to tumor cell vaccines is irradiation to render them unable to propagate in the recipient of the vaccine. Although irradiation causes primarily apoptosis, it can also cause necrosis, which can activate these "danger signals." Alternatively, these "danger signals" signals could be provided by immunological adjuvants such as inflammatory bacteria or bacterial cell-wall products. The two most developed whole-cell vaccines, CanVaxin™ and autologous colon cancer cells (Oncovax™), are administered with bacille Calmette-Guérin (BCG). The complexity of the manipulations necessary to achieve an immune response were highlighted by the earlier work of Hanna (7) in guinea pig models. Hanna's work that suggested that the ratio of viable BCG organisms to tumor cells, procedures for freezing the tumor cells, X-ray treatment of tumor-cells, and vaccination regimen were all important for activity of tumor cell/BCG vaccines.

4. USE OF AUTOLOGOUS OR ALLOGENEIC VACCINES

One question in the development of whole-cell vaccines is whether autologous or allogeneic vaccine should be used. Although autologous (and thus personalized vaccines) are appealing, they are difficult to produce. The small size of many tumor specimens obtained by biopsy can make it difficult to obtain sufficient cells for therapy, especially when multiple immunizations are part of the clinical protocol. In some instances, it is possible to propagate tumor cells *in vitro* to increase the number of cells available, but prolonged cell culture may radically alter the characteristics of the tumor-cells if dominant clones outgrow the other cells present in the tumor sample. More practical has been the development of allogeneic tumor cell banks, although significant regulatory requirements that include detailed characterization of the cellular product and assays to detect adventitious agents remain a challenge. Although allogeneic tumors would not be expected to directly prime immune responses (unless they shared some major histocompatibility complex [MHC] commonality with the host), their allo-antigens

could provide the danger signals needed to activate an immune response. For example, Ward (8) observed that in a murine melanoma model, vaccination of B6 mice with allogeneic K1735 melanoma cells protected against challenge with syngeneic B16 melanoma. This protection could not be improved on by cytokine transfection of the vaccine cells (9). In contrast, K1735 cells administered to syngeneic mice offered no protection against autologous challenge unless the K1735 were transfected with granulocyte-macrophage colony-stimulating factor.

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1. INTRODUCTION

The remarkable advances in biotechnology of the late 20th century created the necessary tools for further defining the principles of tumor immunology and immunotherapy. New and more efficient gene transfer technologies have been developed to enable the expression of specific immune-activating genes at desired levels by tumor cells. The ready availability of recombinant, immune-activating cytokines and chemokines has facilitated assessment of their activity delivered either systemically as a traditional drug, or by gene transfer in preclinical models and clinical trials. Dissection of the molecular mechanisms of T-cell activation has revealed a complex network of signaling pathways that integrate the positive and negative stimuli impinging on the T cell to determine its ultimate functional status. This has created a number of targets for ex vivo and in vivo manipulation to maximize vaccine-activated antitumor immune responses.

2. HISTORY OF GENETICALLY ALTERED TUMOR VACCINES

The rationale underlying the use of genetically modified tumor cells as cancer vaccines dates back to several observations reported in the late 19th and early 20th centuries.

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William Coley used pyogenic bacterial extracts to stimulate antitumor immunity in patients with established soft-tissue sarcomas, reporting marked regression in a significant number of patients (1,2). Chester Southam used the homotransplantation of human tumor cells to investigate differences in immune surveillance between patients with terminal cancer and normal individuals, demonstrating a faster rejection response in healthy subjects than in the cancer patients (3). Prehn and Main demonstrated that irradiation of tumor cells even in the absence of gene transfer or immune adjuvants can enhance the activation of antitumor immunity by inoculated tumor cells (4,5). The results of these investigations laid the groundwork for testing the efficacy of irradiated tumor cells admixed with immune adjuvants composed of pyogenic bacterial extracts derived from mycobacteria (*bacille Calmette-Guérin [BCG]*) or corynebacteria (*Corynebacterium parvum*) in cancer patients (6–10). These early experiments provided evidence of both tumor-specific immunity and clinical responses in patients with melanoma and renal cell carcinoma.

Lindeman and Klein performed the first studies of genetically altered tumor cell vaccines (11). They demonstrated that immunization with influenza virus–infected tumor cell lysates resulted in a systemic immune response protective against a subsequent challenge with wild-type tumor cells. In contrast, immunization with uninfected tumor cell lysates or uninfected lysates admixed with influenza virus were incapable of stimulating the development of protective antitumor immunity. The tumor-specific immune response generated in this strategy was most likely a bystander response that developed in the inflammatory milieu created by the viral infection. Specifically, the viral infection probably upregulated the cell’s antigen-processing machinery, inducing the expression of high levels of both major histocompatibility complex (MHC) class I and MHC class II molecules. This vaccine simultaneously presented processed viral *and* tumor antigens, priming CD8+ and CD4+ T-cell immunity specific for influenza virus and the tumor. These studies provided the proof of principle that genetically altered tumor cells can elicit more robust antitumor immunity than unmodified tumor cells.

After the discovery that bacteria elicit the secretion of inflammatory cytokines, scientists recapitulated Coley’s work in a more defined way by testing the systemic administration of recombinant cytokines such as interleukin-2 (IL-2) and tumor necrosis factor (TNF) to patients with advanced cancers. This resulted in a systemic inflammatory response (SIRS) similar to the septic shock induced by disseminated Gram-negative bacterial infection, with little evidence of antitumor activity (12,13). Subsequent studies utilized lower doses of systemic IL-2 or interferon (IFN)- α to minimize toxicity, demonstrating very low response rates that included the occasionally remarkable clinical response (14,15). In a distinct approach, Forni and colleagues explored the peri-tumoral administration of recombinant cytokines (16). They found that local cytokine delivery resulted in robust leukocyte infiltration at the tumor site without significant systemic toxicity. Moreover, treated animals rejected subsequent tumor challenge, suggesting the development of persistent, systemic antitumor immunity.

This work together established the principles underlying the subsequent development of genetically modified tumor cell vaccines. First, cancer patients typically have a more tepid immune response to unmodified tumor cells than healthy individuals, arguing for the development of strategies designed to overcome immune tolerance and suppression in patients with advanced tumors. Second, an inflammatory or danger signal (17) (such as that provided by bacterial or viral antigens, or recombinant cytokines) can signifi-

cantly enhance tumor-specific immune priming. Finally, localization of the inflammatory signal to the site of antigen delivery *in vivo* maximizes the efficiency of immune priming and minimizes systemic toxicity.

3. ANTIGEN DELIVERY

Antigen delivery is one of the critical parameters of vaccine design. Vaccine formulations based on peptides, proteins, naked DNA, and recombinant viruses all require preexisting knowledge of immunologically active tumor antigens. Tumor cell vaccines are an appealing immunization platform since they can simultaneously deliver a variety of tumor antigens without requiring preexisting knowledge of the most important antigens for immune activation. Also, they have the inherent potential for inducing a polyvalent antitumor immune response. A response targeting multiple tumor antigens is likely to both have greater potency and prevent the outgrowth of antigen-loss variants than an immune response specific for a single antigen only. Along with these advantages, the use of tumor cell vaccines also poses some challenges. Due to concerns that the critical tumor antigens for tumor rejection might be unique to each tumor, early clinical trials utilized autologous tumor cells as the base for vaccine formulation. These studies demonstrated that tumor cell yields are often insufficient to support most immunization protocols. This technical limitation, together with the time and cost involved in developing individualized vaccines, led to interest in the use of allogeneic tumor cells as a more generalizable cancer vaccine platform. This requires that the allogeneic cells deliver shared tumor antigens common to many patients, a concept that has been validated in melanoma and renal cell carcinoma (18–23). Because host professional antigen-presenting cells, not the vaccinating tumor cells, prime the antigen-specific immune response (24,25), there is no need to match the MHC haplotype of the vaccine cells and the patient. Thus, although an allogeneic tumor vaccine is generally restricted to a single tumor type, it is applicable to multiple patients with that tumor histology. An even more broadly applicable approach to the formulation of tumor cell vaccines is the use of genetically modified bystander cells, such as fibroblasts (26) or K562 cells (27). Genes encoding the target of interest are transferred to these cells, which are then admixed with autologous tumor cells. A bystander vaccine is thus generalizable to both a variety of tumor histologies and MHC haplotypes, and is most applicable when large quantities of tumor cells are readily accessible (i.e., by bone marrow harvest or leukapheresis in the hematologic malignancies). The requirement for significant quantities of autologous tumor cells may be overcome by substituting allogeneic tumor cells as the source of tumor antigens in this approach.

4. METHODS OF GENE TRANSFER

Approaches for gene transfer to tumor cells can be broadly classified into those involving plasmid or naked DNA, and those involving viral vectors. Methods for transferring naked DNA into cells include coprecipitation with calcium phosphate (28), the use of high-voltage electric current to produce transient openings in the cell membrane for DNA entry (29), the direct deposition of DNA into the cell by microinjection (30), and the incorporation of DNA into liposomes (31). These methods are moderately efficient for expressing genes transiently in up to 50% of the target cell population, but result in stable, prolonged gene expression in less than 1% of cells. The development of a gene-modified

Table 1. Viral Vectors for Gene Transfer

<i>Virus</i>	<i>Characteristics</i>	<i>Advantages</i>	<i>Limitations</i>
Retrovirus	RNA virus Integrates into genome	Potential for long-term gene expression Potential for pseudotyping to alter tropism	Infects dividing cells only Transgene expression often shut down after transfer
Lentivirus	RNA virus Integrates into genome	Infects nondividing cells Potential for long-term gene expression Accommodates large transgenes	Not yet tested clinically Limited transgene expression
Adenovirus	DNA virus Maintained as an episome	Infects nondividing cells Broad cell tropism Accommodates large transgenes Minimal toxicity	Transient transgene expression Limited by preexisting immunity
AAV	DNA virus Replication requires helper virus Integrated and episomal forms	Infects nondividing cells Broad cell tropism Potential for long-term gene expression Minimal toxicity	Difficult to produce Low packaging capacity
Herpesvirus	DNA virus Integrated and episomal forms	Infects nondividing cells Broad cell tropism Accommodates large transgenes Potential for long-term gene expression	Not yet tested clinically Potential for immune responses that limit transgene expression
Baculovirus	DNA virus Integrates into genome	Infects nondividing cells Broad cell tropism Minimal toxicity No replication in mammalian cells	Sensitive to complement Preclinical experience limited Not yet tested clinically

Note. A summary of the characteristics, advantages, and limitations of various viral vectors for gene transfer.

tumor cell line that expresses consistent levels of the target gene over time requires the selection of cells that have stably integrated the gene into their genome. If a gene encoding a hydrolytic enzyme for a toxic compound (i.e., an antibiotic such as hygromycin) is included in the gene transfer, cells that have stably integrated the transferred DNA will survive growth in culture media containing the compound, whereas unmodified cells will not survive the selection. Although this process will increase the number of genetically modified cells to virtually 100%, it is possible to lose subpopulations of cells that might express antigenic targets important in the antitumor response during the selection process. The advantage of these gene transfer strategies is their safety, with little to no risk of generating recombinants *in vivo* that are potentially detrimental to the patient.

By far the most efficient method for gene transfer into primary mammalian cells or cell lines is the *ex vivo* or *in vivo* use of viral vectors. A variety of viral vectors have been developed for gene transfer, including lentiviruses (32), retroviruses (32), adenoviruses (33), adeno-associated viruses (AAV) (34), herpesviruses (33), and baculoviruses (35) (Table 1). These vectors infect their host cell by binding to specific cell-surface receptors. Most viral vectors are constructed to provide regulatory signals for mRNA transcription, translation, splicing, and polyadenylation, resulting in highly efficient expression of the gene product. Depending on the vector system chosen, the risks associated with viral-mediated gene transfer are significantly greater than gene transfer with plasmid DNA (36). They include damage or death to the infected cell, activation of latent viruses or proto-oncogenes present in the host genome, the transformation of replication-defective viral vectors to replication-competent viruses by recombination with host gene sequences, and the development of viral pseudotypes with altered cell tropism by the complementation of viral vector sequences with viral sequences endogenous to the infected host cell. Potential complications associated with the *in vivo* use of viral vectors to achieve gene transfer include diminished efficacy due to preexisting neutralizing antiviral immunity, and the induction of a systemic inflammatory response (SIRS) with or subsequent to viral infusion that can be life threatening.

5. MECHANISMS OF ANTITUMOR IMMUNITY

Because tumor cells arise endogenously, they are surveyed by the immune system in a noninflammatory environment. This is thought to result in immune tolerance, and the goal of gene-modified tumor vaccines is to overcome established mechanisms of immune tolerance by presenting relevant tumor antigens in an inflammatory context. Current approaches to tumor cell vaccine design are based on manipulating T cells or antigen-presenting cells (APCs) to recapitulate an inflammatory environment. T cells can be activated directly by either increasing the MHC:peptide density presented by the vaccine, or genetically modifying the vaccinating tumor cells to express costimulatory molecules that provide a second activation signal to the T cell. This second signal, provided by cytokine gene transfer or the transfer of genes encoding costimulatory molecules, synergizes with the activation signal generated by engagement of the T-cell receptor (TCR)-MHC:Ag complex. T cells can also be activated indirectly by genetically modifying the tumor vaccine cells to provide sustained local delivery of cytokines that recruit professional APCs to the site of antigen deposition *in vivo*. In both cases, activated, professional APCs initiate CD4+ and CD8+ T-cell responses by capturing, endocytosing, and processing tumor antigens released by cancer cells. Professional APCs simulta-

neously present tumor antigens to both CD4+ and CD8+ T cells in the context of MHC class II and MHC class I respectively, thus cross-priming the antigen-specific immune response (25,37). Activated CD4+ T cells initiate and amplify the CD8+ T-cell response directly by providing stimulatory cytokines, and indirectly by upregulating a variety of costimulatory molecules on the APCs that provide a second signal for T-cell activation (38). Activated CD8+ T cells then acquire the potential to lyse tumor cells (39). Notably, in the absence of a danger or inflammatory signal, critical costimulatory molecules are not upregulated on the surface of the APCs, resulting in downregulation of the T-cell response (40). The influence of the inflammatory milieu at the time of immune priming and activation thus has clear implications for the clinical development of vaccine-based approaches to cancer treatment. Detailed understanding of these complex pathways of immune activation has identified a number of appealing targets for study in the development of gene-modified tumor vaccines.

6. MHC GENE TRANSFER

Two of the most important determinants of T-cell responses as determined by the peptide:MHC/TCR interaction are the TCR affinity and the peptide:MHC ligand density. Increasing syngeneic or allogeneic MHC class I gene expression by gene transfer was thus one of the earliest approaches for enhancing the immunogenicity of tumor cells. The introduction of H-2K MHC molecules can abrogate the metastatic potential of some tumor cells, an observation attributed to increased antigen presentation to tumor-specific CD8+ cytotoxic T cells (CTLs) *in vivo* (41). In contrast, augmenting MHC class I expression resulted in diminished natural killer (NK)-cell activity and enhanced tumorigenicity in a lymphoma model (42). This suggests that altering the antigen presentation potential of a tumor cell derived from a professional APC (i.e., a B cell) might be counterproductive. The delivery of allogeneic MHC molecules is analogous to immunization with a foreign protein, and elicits a potent immune response. Contrary to hypotheses that foreign MHC molecules might induce a dominant allogeneic human leukocyte antigen (HLA) response that impairs the development of antitumor immunity, the codelivery of allogeneic MHC class I genes and tumor-specific antigens also enhances tumor-specific immune responses *in vitro* and *in vivo* (24,25,43,44). Furthermore, the introduction of allogeneic MHC molecules into tumor cells can result in a T-cell response characterized by therapeutic cytokine deviation (45). Specifically, T cells activated by nonimmunogenic syngeneic tumor cells exhibit an ineffective type 2 cytokine profile, secreting interleukin-4 (IL-4). In contrast, T cells activated by tumor cells genetically modified to express allogeneic H-2^b display a therapeutic type 1 cytokine phenotype, secreting interferon- γ (IFN- γ). Only these latter T cells display antitumor activity upon adoptive transfer *in vivo*.

7. COSTIMULATORY MOLECULES: CD80, CD86, AND CD40

It is now well established that effective T-cell activation requires the antigen-dependent signal (signal 1) provided by engagement of the TCR with the peptide:MHC complex, and the antigen-independent signal (signal 2) provided by accessory molecules for T-cell stimulation (46). Although the antigen-independent signal was originally thought to result from engagement of CD80 (B7-1) with its receptor CD28, it has become clear that at least three different types of molecules participate in T-cell costimulation (38). These are the B7 family, the tumor necrosis receptor family, and the cytokines (Table 2).

Table 2
Costimulatory Molecules for T-Cell Activation

	<i>T-Cell Coreceptor</i>	<i>Signal</i>	<i>Reference</i>
B-7 Family			
B7-1/B7-2	CD28	+	47,48
B7-1/B7-2	CTLA4	-	47,48
B7RP-1	ICOS	+	47
B7H-3	unknown	+	38
B7H-1/B7DC	PD-1	-	58
B7H-1/B7DC	unknown	+	38
TNF Receptor Family			
4-1BBL	4-1BB	+	62
CD27L	CD27	+	63
OX40L	OX40	+	64
Light	Light-R	+	65
CD40L	CD40	+	66

Note. There are three types of molecules for T-cell costimulation in the activation of immunity: the B-7 family, the tumor necrosis factor receptor (TNFR) family, and the cytokines. Shown are the various members of the B-7 and TNFR families, their cognate T-cell co-receptors, and the type of signal transduced upon receptor engagement. Several cytokines, including interleukin-2, interleukin-12, and interleukin-18, represent a third group of immunomodulatory molecules that can also contribute to T-cell activation (not shown) (38).

It is the integration of all these signals that determines the resulting strength of signal 2 for T-cell activation.

7.1. The B7 Pathways

The B7 family consists of at least six members (47). The original costimulatory B7 molecules identified were CD80 (B7-1) and CD86 (B7-2). CD86 is thought to play an integral role in the priming of a cell-mediated immune response, whereas CD80 is thought to maintain and/or modulate that response (48). Each molecule can bind to CD28, an activating receptor (49), and to CTLA4, an inhibitory receptor that downregulates T-cell responses by mechanisms that remain undefined (50). Because the remaining members of the B7 family are so new, most gene transfer studies reported to date have focused on CD80 and CD86. Multiple studies have shown that CD80-modified syngeneic tumors are rejected as compared to unmodified syngeneic tumors (51–54). Animals that rejected those tumors were subsequently protected from rechallenge with wild-type tumor. It is important to note that CD80 modification alone was not always sufficient to induce antitumor immunity. Rather, efficient tumor rejection sometimes required a second genetic modification of the tumor cells, either with a potent tumor antigen like HPV-16 E7 (51), or with MHC class II molecules (52). The hypothesis underlying these studies is that CD80 expression by the genetically modified tumor cells essentially converts the tumor cell to an APC (55). Signal 1 is thus provided by tumor antigen bound to endogenous syngeneic MHC class I, and signal 2 by CD80. More detailed analysis revealed that direct antigen presentation by the modified tumor cells occurred inefficiently, requiring repeated immunization to expand the directly primed CD8+ CTL (56). In fact, CD80

expression renders some tumor cells more susceptible to lysis by NK cells (57), thus facilitating the uptake and processing of antigen by host APCs. Thus, the dominant mechanism of CTL priming by CD80-modified tumor cells is indirect, through the uptake and presentation of tumor antigen by bone marrow-derived professional APCs (56). Clinical trials testing CD80-modified autologous renal cell carcinoma (58) and CD80-modified allogeneic breast carcinoma tumor vaccines (59) have been reported, both with some preliminary evidence of bioactivity as measured by delayed-type hypersensitivity (DTH) to autologous tumor.

The ability of tumor cells genetically modified to express newer members of the B7 family to boost antitumor responses is the subject of current study (60). Interestingly, it was recently reported that tumor cell-associated B7-H1/B7-DC can promote antigen-specific T-cell apoptosis through binding inhibitory receptors distinct from the known inhibitory receptor PD-1 (61). The expression of B7-H1/B7-DC in this study was exclusive to cells of the macrophage lineage and transformed tumor cells, with no expression in normal human tissues. Expression of B7-H1/B7-DC on mouse P815 tumor cells increased apoptosis of activated tumor-specific CTL, and augmented the growth of normally immunogenic CD80-expressing P186 tumors *in vivo*. These observations suggest that tumor-specific expression of B7-H1/B7-DC could be a mechanism of immune evasion, and suggest complex immunoregulatory functions for B7-H1/B7-DC pathways, somewhat analogous to CD80/CD28 and CD80/CTLA4.

7.2. The CD40/CD40L Pathway

CD40 is a member of the tumor necrosis factor receptor (TNFR) family (62–65) broadly expressed on antigen-presenting cells (APCs), B cells, epithelial cells, and endothelial cells (66). Engagement of CD40 on APCs by its ligand CD40L (CD154) on responding T cells augments the resulting immune response (66). Exogenous activation of the CD40 pathway by agonist antibodies is capable of substituting for T-cell help (67), and augments both humoral and CTL responses (68). Manipulation of the CD40 pathway by engineering chronic lymphocytic leukemia (CLL) cells to overexpress CD40L by adenoviral gene transfer induces the expression of multiple costimulatory molecules. This augments the antigen-presenting capacity of both CD40L-modified CLL cells and unmodified bystander CLL cells *in vitro*, enabling both of them to prime CTL specific for autologous CLL (69). CD40L modification also augments the antigen-presenting capacity of human multiple myeloma cells *in vitro* (70,71). The ability of CD40L-modified cells to induce immune-mediated tumor regression *in vivo* has been demonstrated in animal models of B-cell lymphoma (72), melanoma (73), colon carcinoma (73), and bladder carcinoma (74). One clinical trial tested the infusion of autologous CLL modified with a replication-defective adenoviral vector delivering CD40L in 11 patients with progressive intermediate- or high-risk CLL (75). This therapy increased the expression of CD80, CD86, ICAM-1, and CD95 on uninfected CLL *in vivo*. Treated patients developed high plasma levels of the T helper type 1 cytokines IL-12 and IFN- γ , increases in absolute T-cell counts over 240%, and increased numbers of CLL-specific, IFN- γ -producing T cells by ELISPOT. These immune responses correlated with clinical responses as measured by reduced lymphocyte counts and lymph node size. Together, these data support the further investigation of CD40L-modified tumor cell vaccines for the treatment of both hematologic and solid-tumor malignancies.

Table 3
Cytokine Genes Transferred to Tumor Cells for Vaccine Development

<i>Cytokine</i>	<i>Tumor Regression in Preclinical Models</i>	<i>Immune Mediators</i>	<i>Tested Clinically</i>	<i>Reference</i>
Interleukin-2	yes	CD8+, NK cells lymphocytic infiltrates	yes	76–80
Interleukin-4	yes	CD8+ T cells, partial CD4+ T cells macrophage/eosinophil infiltrates	yes	81–83
TNF	depends on tumor	N/A	no	84–86
Interferon- γ	depends on tumor	upregulation of MHC class I and II	no	87–89
Interferon- α	yes	CD8+ T cells	no	90
G-CSF	no	macrophage infiltrates	N/A	92
Interleukin-7	yes	CD4+ T cells, macrophages	yes	93–96
Interleukin-12	yes	CD8+ T cells, partial CD4+ T cells	yes	97–100
Flt-3 Ligand	yes	unknown	no	101, 102
GM-CSF	yes	CD8+ T cells, CD4+ T cells macrophage/DC/ eosinophil infiltrates	yes	83, 116–118, 124–126

Note. A summary of cytokine genes that have been transferred to tumor cells for preclinical and clinical study in cancer vaccine development. Abbreviations: TNF, tumor necrosis factor; MCP-1, macrophage chemotactic protein-1; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; NK, natural killer; MHC, major histocompatibility complex; DC, dendritic cells.

8. CYTOKINE-MODIFIED TUMOR VACCINES

The ability of bacterial products to elicit inflammatory cytokines underlies the antitumor activity observed with the administration of bacterial extracts as immunologic adjuvants. The ability of locally delivered BCG or recombinant cytokines to enhance antigen-specific immunity led to the testing of cytokine-secreting tumor vaccines as a strategy for delivering relevant tumor antigens in the context of immune-activating cytokines. A number of cytokines, including IL-2 (76–80), IL-4 (81–83), TNF (84–86), IFN- γ (87–89), IFN- α (90), MCP-1 (91), G-CSF (granulocyte colony-stimulating factor) (92), IL-7 (93–96), IL-12 (97–100), Flt-3 ligand (101, 102), and GM-CSF (granulocyte-macrophage colony-stimulating factor) (83) have been tested in preclinical gene transfer studies and/or clinical trials (summarized in Table 3). This discussion will focus on the use of IL-12, Flt-3 ligand, and GM-CSF gene transfer, because the mechanisms by which tumor cells that secrete these cytokines activate antitumor immunity have been most thoroughly studied.

8.1. Interleukin-12

IL-12 is a pleiotropic cytokine that links the innate and adaptive arms of the immune response (100). IL-12 mediates a potent antitumor effect secondary to the activation of NK and CD8+ T cells and the resulting IFN- γ secretion. IFN- γ then feeds back on the IL-12-producing APCs, augmenting their secretion of IL-12 and providing a powerful amplification mechanism. The antitumor effect of IL-12 is in part the reflection of IFN- γ -mediated inhibition of angiogenesis. IFN- γ stimulates the production of IP10 and Mig, two chemokines that bind to the chemokine receptor CXCR3 to block angiogenesis.

IL-12-secreting tumor vaccines elicit antitumor immunity in a variety of preclinical models, including mammary carcinoma, colon carcinoma, and melanoma. IL-12-transduced autologous tumor vaccines elicit CD8+ T-cell-dependent antitumor immunity capable of curing metastatic disease in the C26 model of colon cancer (103). The antitumor effect of these vaccines, which secreted pg levels of IL-12, was enhanced by CD4+ T-cell depletion (104). In the absence of IFN- γ (i.e., IFN- γ knockout mice), this immunity was dependent on CD4+ T cells, neutrophils, and GM-CSF (105,106). Interestingly, a direct comparison of mechanisms of antitumor immunity mediated by IL-2-secreting C26 cells as compared to IL-12-secreting C26 cells revealed that only IL-12-modified C26 vaccination was curative despite the equivalent induction of CTL (107). This was attributed to the ability of IL-12 to shift antibody production to complement-fixing IgG2a antibodies capable of lysing C26 target cells.

Importantly, activation of the CD80/CD28 costimulatory pathway and the IL-12 pathway are synergistic signals for T-cell activation as measured by IFN- γ production and proliferation in both murine (108) and human systems (109). The systemic administration of IL-12 to mice inoculated with CD80-modified SCK mammary carcinoma cells resulted in cure of 74% of the mice (108). Moreover, these surviving mice rejected subsequent challenge with wild-type SCK cells in the setting of active, systemic antitumor immunity dependent on CD4+ T cells, CD8+ T cells, and IFN- γ production. Interestingly, IL-18 also synergizes with IL-12 to induce IFN- γ production in preclinical gene transfer experiments. SCK mammary carcinoma cells genetically engineered to secrete either IL-12 or IL-18 failed to protect against subsequent challenge with unmodified SCK cells, but injection of admixed IL-12- and IL-18-secreting cells protected 70% of mice from tumor challenge, and cured 30% of mice with preestablished tumor (110). The surviving mice were not protected from a later tumor challenge, arguing against the induction of durable antitumor immunity. This, together with matrigel plug experiments demonstrating the inhibition of angiogenesis, strongly supports the antiangiogenic activity of IL-12 as the primary mechanism of antitumor activity in these experiments. IL-12 thus exerts antitumor activity through a variety of mechanisms, only some of which are manifestations of adaptive immunity. Early after the systemic administration of IL-12 with tumor vaccine, the potent antitumor effect appears to be secondary to inhibition of angiogenesis rather than the induction of antitumor immunity. Adaptive immunity develops later, and it may be possible to develop IL-12-based immunotherapies that capitalize on both aspects of this cytokine's antitumor efficacy.

8.2. Flt-3 Ligand

The pivotal role of dendritic cells (DCs) as professional APCs in the induction of antitumor immunity relates to their ability to take up, process, and present relevant anti-

gens in the context of both MHC class I and MHC class II, thus activating both CD8+ and CD4+ T-cell immunity (111). There has been great interest in enhancing the numbers and/or function of DCs involved in priming antitumor immunity *in vivo*, either by ex vivo manipulation of DCs or by the recruitment and modulation of DCs *in vivo*. The two most potent cytokines eliciting DC mobilization, proliferation, and maturation are GM-CSF and Flt-3 ligand (102,112). The systemic administration of Flt-3 ligand results in the marked expansion of DCs, and one study demonstrated the ability of Flt-3 ligand-modified cells to induce durable antitumor immunity protecting up to 90% mice from challenge with unmodified parental cells (101). Both tumor-specific T cells and NK cells have been implicated in this effect. GM-CSF and Flt-3 ligand-modified tumor vaccines were recently compared head-to-head in a B16 melanoma model (102). Both vaccine types induced a marked increase in CD11c+ cells locally and systemically. Flt-3 ligand induced the differentiation of both lymphoid-type (CD8α+ and CD11b-) and myeloid-type (CD8α- and CD11b+) DC and a T-cell response skewed toward the type 1 profile. In contrast, GM-CSF-secreting vaccines generated myeloid-type DCs expressing high levels of B7-1 and CD1d and a T-cell cytokine profile that was not skewed toward either the type 1 or type 2 profiles. Importantly, the antitumor activity of GM-CSF-modified tumor vaccines was vastly superior to that of Flt-3 ligand-secreting vaccines. This may be explained in part by observations that CD8α- DCs more effectively take up particulate antigen than CD8α+ DCs (113).

8.3. Granulocyte-Macrophage Colony-Stimulating Factor

GM-CSF emerged early in tumor vaccine development as a highly promising cytokine for the development of gene-modified tumor vaccines. In a seminal study, GM-CSF was identified as the most potent cytokine for inducing systemic antitumor immunity in the poorly immunogenic B16F10 melanoma model (83). This study directly compared IL-1, IL-2, IL-4, IL-5, IL-6, GM-CSF, IFN- γ , and TNF- α delivered by retroviral transduction to B16F10 melanoma cells and demonstrated the clear superiority of GM-CSF for inducing antitumor immunity. The level of immunity was also greater than that elicited by unmodified irradiated cells alone or admixed with the bacterial adjuvants BCG or *Corynebacterium parvum*. GM-CSF-modified tumor cells activate CD8+ and CD4+ T-cell-dependent antitumor immunity capable of curing mice of preestablished small tumor burdens of various histologies. As suggested earlier, GM-CSF recruits and activates DCs to cross-prime the antigen-specific immune response (24), a process initiated by local secretion of the chemokine MIP1- α (114,115). The cellular infiltrates induced by paracrine GM-CSF secretion at the vaccination site consist predominantly of macrophages, DCs, and eosinophils early after inoculation, with a shift to mature lymphocytes and eosinophils 1 wk later (116,117). These vaccines stimulate a broad immune response initiated by both T helper type 1 and T helper type 2 cells (118–120). Although most investigations of mechanism have focused on the adaptive immune response, GM-CSF-secreting vaccines also activate innate immunity mediated by the production of antiangiogenic factors and chemokines secreted by activated macrophages (115,121).

The superiority of GM-CSF-modified tumor vaccines in murine models has led to great interest in translating this vaccination strategy to cancer patients. An aspect of tumor vaccine development that is often overlooked is the careful delineation in preclinical models of the vaccine formulation and administration parameters critical for the most robust biologic effect. The important variables to consider in the development of GM-

CSF-secreting vaccines have been relatively well established. First, the number of tumor cells used in immunization clearly reflects the amount of antigen delivered; both preclinical and clinical studies suggest that increasing the number of vaccine cells augments the potency of systemic antitumor immunity (122). Second, the spatial distribution of vaccine over multiple lymph node regions also enhances vaccine activity compared to administration limited to a single lymph node basin (123). Third, the concentration of cytokine produced by the vaccinating cells is critical. The optimal amount varies somewhat depending on the cytokine, but in general ng quantities are desirable. For GM-CSF-secreting vaccines, a minimum production of 35 ng/10⁶ cells/24 h for 4–5 d in vivo is required (122). Fourth, methods must be developed that ensure that the inoculated cells do not establish tumors and cause morbidity, but that do not diminish vaccine efficacy. For GM-CSF-secreting tumor vaccines, irradiation is an effective means of activating the apoptotic program, while allowing the cells to remain metabolically active to secrete GM-CSF for the requisite 4–5 d in vivo (122). Finally, the optimal route of administration is also important to determine. Comparison of intravenous, intramuscular, subcutaneous, and intraperitoneal routes revealed the subcutaneous and intramuscular routes to be better. Other routes worthy of investigation include intratumoral, intralymphatic, and intradermal.

Tumor cells modified to secrete GM-CSF have been tested clinically as autologous, allogeneic, and bystander formulations in a variety of cancers (Table 4). These include melanoma (118), renal cell carcinoma (116), pancreatic carcinoma (117), and several other poorly immunogenic solid tumors (124–126), as well as multiple myeloma and acute myelogenous leukemia. These trials have demonstrated GM-CSF-secreting vaccines to be safe, with side effects limited primarily to erythema and induration at the vaccination site (Table 5). These trials in the aggregate have also provided preliminary evidence of vaccine bioactivity. Several parameters important for the clinical application of GM-CSF-secreting vaccines were confirmed by the trial testing two allogeneic pancreatic carcinoma cell lines secreting GM-CSF at levels of 220 ng/10⁶/24 h in patients with stage II and III pancreatic cancer after pancreaticoduodenectomy (117). Patients were vaccinated immediately after surgery and before adjuvant chemoradiation; patients who remained disease-free at the completion of adjuvant therapy were eligible to receive three additional vaccinations at monthly intervals. This study was the first to escalate the vaccine cell dose to 5 × 10⁸ cells. Four of five patients who received 5 × 10⁸ cells had detectable levels of serum GM-CSF that recapitulated the pharmacokinetics observed in preclinical models—no patient at the lower vaccine cell-dose levels had detectable levels of serum GM-CSF. Clinically significant DTH responses (>1.0 cm) to dissociated autologous pancreatic tumor cells developed in one of three and two of four patients who received 1 × 10⁸ and 5 × 10⁸ vaccine cells respectively. These three patients remained free of disease for over 2 yr, suggesting a potential survival benefit. This trial thus defined 5 × 10⁸ cells as a safe and bioactive dose for testing in other tumor types. A 60-patient efficacy trial of similar design is now actively accruing.

9. CHEMOKINE-MODIFIED TUMOR VACCINES

Chemokines are a large family of molecules with pleiotropic biological activity secreted by virtually all cells, including tumor cells (127). Although they were originally defined by their ability to elicit the directional migration of leukocytes, chemokines are

Table 4
Clinical Trials of GM-CSF-Secreting Tumor Vaccines

Principal Investigator	Trial Type	Disease	Cell Type	Vector	Number of Treated Patients	DTH Response ≥1 cm	Antitumor Response
Simons ^a	Phase I	renal cell cancer	autologous	retrovirus	7	57%	11%
Soiffer ^b	Phase I	melanoma	autologous	retrovirus	29	100%	7%
Simons ^c	Phase I	prostate cancer	autologous	retrovirus	8	75%	NR
Jaffee ^d	Phase I	pancreatic cancer	allogeneic	plasmid	14	21%	21%
Simons ^e	Phase II	prostate cancer	allogeneic	retrovirus	96	NR	*
Nemunaitis ^f	Phase I/II	lung cancer	autologous	adenovirus	12	NR	25%

Note. A summary of clinical trials of GM-CSF-secreting tumor vaccines reported to date. NR = not reported. ^aSimons et al. treated 7 out of 16 patients with an autologous GM-CSF-secreting renal cell cancer vaccine; the other 9 patients were vaccinated with unmodified autologous tumor cells (116). Four of the seven patients developed clinically significant DTH responses to autologous tumor. The one patient who developed the largest DTH response developed regression of multiple pulmonary metastases. ^bSoiffer et al. treated 29 patients, 100% of whom developed DTH responses to autologous tumor (118). They observed one partial response, one mixed response, and three minor responses. ^cSimons et al. treated eight patients with metastatic prostate cancer, and observed clinically significant DTH reactions in six of eight patients. Tumor responses were not reported. ^dJaffee et al. incorporated vaccination into an aggressive adjuvant protocol including chemotherapy and radiation for patients with high-risk pancreatic cancer; 14 patients were vaccinated (117). Three of the 14 patients receiving the highest vaccine cell doses developed clinically significant DTH to autologous tumor, and remain disease-free about 4 yr from initial surgery. ^eSimons et al. treated 96 patients in a multicenter phase II trial of a retrovirally transduced autologous prostate cancer vaccine. DTH results have not been reported (125). The interim analysis reported one partial response and one complete response. ^fNemunaitis et al. reported preliminary results of a phase I/II trial of 80 patients with either early or advanced non-small-cell lung cancer vaccinated with adenovirus-transduced autologous tumor (126). They reported an interim analysis of 12 patients, and reported one complete remission, one mixed response, and one patient with stable disease. The details of DTH reactivity were not reported. See Table 5 for a compilation of the toxicities observed in clinical trials reported for all GM-CSF-secreting vaccines tested to date.

now known to play roles in angiogenesis, metastasis, and primary tumor growth (128). Importantly, chemokine–ligand receptor interactions are emerging as an additional pathway of costimulation to increase the effector functions of both T cells and NK cells (128). Differential chemokine responsiveness is a determinant of the differentiation status of CD4+ T helper cells and memory T cells. T helper type 1 cells preferentially express CCR3 and CCR5, whereas CCR4 and CCR8 expression are more characteristic of T helper type 2 cells (129–131). Memory T cells are characterized by CCR7 expression. The role of chemokines in attracting APCs or T cells suggests that their rational inclusion in the formulation of genetically modified tumor cell vaccines might augment the intensity of the immune response induced. For example, the use of chemokines to skew the immune response toward the type 1 cytokine profile at the level of immune priming might enhance vaccine efficacy. Consistent with this, tumor cells engineered to

Table 5
Clinical Toxicities of GM-CSF-Secreting Tumor Vaccines

<i>Local Toxicity^a</i>	<i>Systemic Toxicity</i>
1. Induration ^b	1. Pruritus distant from the vaccine site
2. Erythema ^b	2. Urticaria
3. Pruritus ^b	3. Systemic skin rash/Grover's syndrome
4. Tenderness ^b	4. Arthralgia/myalgia
5. Recall induration ^b	5. Fever
6. Ulceration ^b	6. Fatigue
7. Lymphedema of the vaccinated limb	7. Chills, malaise
	8. Other ^c

Note. A summary of toxicities observed in all clinical trials of GM-CSF-secreting tumor vaccines reported to date. Over 150 patients have been treated, about 40% with autologous vaccines and 60% with allogeneic vaccines. Over 450 vaccines have been administered. ^aThe most common toxicity associated with GM-CSF-secreting vaccines is local skin reaction consisting of self-limited induration, erythema, and sometimes pruritus. ^bDenotes toxicity at the vaccine site. ^cOne patient in the allogeneic pancreatic tumor vaccine trial developed a clinical syndrome of thrombocytopenia, hemolytic anemia, and seizure consistent with thrombotic thrombocytopenic purpura (TTP). This was attributed to the mitomycin C in the adjuvant chemotherapy regimen.

secrete CCL5/RANTES (which binds to CCR3 and CCR5, chemokine receptors expressed on T helper type 1 cells) (132) or CCL3/MIP1 α (133) elicit robust infiltrates of T cells that exert an antitumor effect *in vivo*. Alternatively, modulation of the DC—naïve T-cell interaction at the site of immune priming might enhance vaccine efficacy. Secondary lymphoid tissue chemokine (SLC) is normally highly expressed in secondary lymphoid tissues, and directs the migration of DCs from the skin to the draining lymph nodes (134,135). An important role for SLC in the priming of naïve T cells by DCs has been suggested (134). Supporting the incorporation of SLC into gene-modified tumor vaccine formulations, murine C26 colon carcinoma cells engineered to secrete SLC are less tumorigenic than wild-type cells, with antitumor effects mediated in part by CD8+ T cells (136). Finally, it has recently been demonstrated that the intensity of the vaccine-activated antitumor response is directly proportional to its efficacy (137). It is thus attractive to consider codelivering chemokines to attract the desired type of immune cell to the vaccine site, and immunomodulatory cytokines like IL-2 and GM-CSF that serve to amplify the induced immune response. The concept of a two-hit strategy for immune priming is illustrated by the examples provided below.

9.1. Stromal Cell-Derived Factor (SDF)-1

Stromal cell-derived factor 1 (SDF-1) is a chemokine constitutively produced by stromal cells that plays a key role in the developmental patterning of several tissues (138,139). It is pro-angiogenic, and a potent chemotactic factor for T cells and pre-B lymphocytes. It is costimulatory for CD4+ T-cell activation, priming resting T cells for TCR- and cytokine-mediated stimulation (140). SDF-1 also functions as a survival factor for CD4+ T cells, downregulating pro-apoptotic genes, and increasing transcription of genes related to cell survival (141). Several studies have examined the antitumor activity of SDF-1 ectopically expressed in the tumor microenvironment. The activity of SDF-1

is dose-dependent, with MethA fibrosarcoma or HM-1 ovarian carcinoma cells expressing high levels of SDF-1 (90 ng/mL or 55 ng/mL respectively) resulting in negligible tumor regression (142). The combination of SDF-1 and either IL-2 or GM-CSF resulted in a marked tumor regression associated with the development of a T helper type 1 immune response and IFN- γ secretion. In contrast, myeloid leukemia cells or B16F1 melanoma cells expressing low levels of SDF-1 (1.6 or 2.6 ng/mL, respectively) did induce tumor regression, and elicited CD4+ T cell-dependent systemic immunity capable of rejecting a tumor challenge when low numbers of vaccinating cells were used (142). Interestingly, this dose-dependent dichotomy in the development of effective antitumor immunity may relate to observations that high levels of SDF-1 actually reverse *in vivo* antigen-induced T-cell migration away from the antigenic stimulus (144).

9.2. Lymphotactin

Lymphotactin is a chemokine that can induce the migration of T cells and NK cells without influencing the chemotaxis of monocytes or granulocytes. In one study, lymphotactin-modified multiple myeloma cells displayed rapid and complete regression with associated infiltrates of CD4+ and CD8+ T cells and a type 1 cytokine response; unmodified myeloma cells gave rise to 100% tumor incidence (145). The response was also associated with the infiltration of significant numbers of neutrophils, implicating both innate and adaptive immunity in the antitumor effect. Similar studies compared irradiated A20 lymphoma cells admixed with fibroblasts genetically modified to secrete lymphotactin, lymphotactin and IL-2, or lymphotactin and GM-CSF (146). Little antitumor activity was generated by the vaccine formulation delivering lymphotactin alone. In contrast, combined expression of lymphotactin and IL-2 resulted in the marked infiltration of the tumor cells with both CD4+ and CD8+ T cells, and significant regression of established tumor. The combination of lymphotactin and GM-CSF also conferred antitumor activity compared to lymphotactin alone, but was less effective than lymphotactin and IL-2. These studies suggest that lymphotactin attracts T cells to the site of immune priming, where the local secretion of IL-2 serves to expand the activated, tumor-reactive T-cell population to the therapeutic levels required for an antitumor effect *in vivo*.

10. CONCLUSIONS

The study of gene-modified tumor cells has resulted in significant insight into the regulatory mechanisms underlying immune tolerance and the development of effective antitumor immunity. A number of novel targets for gene transfer have been identified and are under active investigation. Early clinical trials have demonstrated the administration of gene-modified tumor vaccines to patients with advanced cancers to be safe, with complications primarily limited to local side effects related to vaccination. Several clinical trials have suggested the potential for clinical benefit, and phase II trials that may provide clinical proof of principle are currently under way. It is clear that the development of second-generation tumor vaccines should include strategies for maximizing the intensity of the antitumor immune response induced, through either the rational formulation of the vaccines themselves or combinatorial therapy with drugs that target critical components of the pathways regulating immune responses. It is also clear that conducting clinical studies that are based on relevant preclinical modeling, and the translation of pertinent clinical findings back to the laboratory for further study, is essential to the effective development of gene-modified tumor vaccines.

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19

Hapten-Modified Tumor Vaccines

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1. DISCOVERY OF HAPTENS

Haptens are tiny lights that illuminate the dark recesses of the immune system. They were discovered by Karl Landsteiner, who used haptens to explore the breadth and fine sensitivity of antibody responses. Landsteiner (1) worked with a variety of simple chemicals, including nitrophenyls and phenyl arsonates, that were incapable of inducing an immune response by themselves, but became immunogenic when they were attached covalently to a protein carrier. He coined the term “hapten” from the Greek *hapttein*, meaning to fasten.

Landsteiner made what was at the time an astounding observation: Rabbits immunized with a haptenized protein produced three sets of antibodies: (a) to the hapten itself, (b) to the carrier protein, and (c) to hapten–protein conjugate. The antihapten antibodies were highly specific in that they did not react to structurally different molecules of the same origin. For example, antibodies elicited by immunization with *meta*-aminobenzene sulfonate showed minimal reactivity to *para*-aminobenzene sulfonate and no reactivity to aminobenzene arsonate or aminobenzene carboxylate (2). These experiments undermined the “almost dogmatic belief...that a special chemical constitution, peculiar to proteins and not even to all of them, is necessary for the production of antibodies” (1).

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2. BREAKING TOLERANCE WITH HAPten-MODIFIED PROTEINS

Weigle extended these observations to proteins that were not immunogenic in their native state. Rabbits that had been rendered tolerant to bovine serum albumin (BSA) by neonatal injections of this protein failed to produce anti-BSA antibody even after injection with Freund's adjuvant. In contrast, unresponsive rabbits injected with BSA conjugated to sulfanilic acid (SA) produced antibody not only to SA-conjugated BSA but to native BSA as well (3). Thus immunization with a hapten-modified protein could break established immunological tolerance to that protein.

Even more surprising was Weigle's observation that hapten conjugation could break natural tolerance (3). The injection of rabbits with homologous thyroglobulin in incomplete Freund's adjuvant produced, as expected, little or no antibody to thyroglobulin. However, rabbits injected with thyroglobulin that had been modified with arsanilic acid plus SA or with trinitrophenyl (TNP) produced precipitating antibody to both modified and native thyroglobulin. Moreover, some of the rabbits developed histological evidence of autoimmune thyroiditis. Once the animals had been immunized, the height of their antibody titers and the severity of the thyroiditis could be increased by administering booster injections of native thyroglobulin.

3. T-CELL RESPONSE TO HAPTENS

As the physiology and biochemistry of T lymphocytes was elucidated, it became clear that hapten-modified proteins also elicited a T-cell response. Guinea pigs that were sensitized by topical application of 1-chloro-2,4-dinitrobenzene (DNFB) developed lymphocytes that proliferated when cultured with dinitrophenyl (DNP)-conjugated syngeneic lymphocytes (4). It was not possible to show that these DNP-reactive lymphocytes were T cells until the availability of antisera (against the so-called θ antigen) that specifically killed T cells (5). In a seminal and probably controversial paper, Rubin and Wigzell reported that DNFB-sensitized mice generated splenic and lymph node lymphocytes that provided help to B cells obtained from mice immunized with BSA when the readout was the *in vitro* production of antibody to DNP-BSA. These helper lymphocytes were, in fact, T cells, as demonstrated by absence of surface anti-IgM (immunoglobulin) and their sensitivity to anti- θ antibody. The same phenomenon could be demonstrated in the murine antibody response to *murine* serum albumin (MSA), but in this case the helper T cells were not specific for DNP but for the "new antigenic determinants" on MSA produced by the DNP conjugation (6).

Subsequently, Shearer demonstrated that cell-mediated cytotoxicity could be induced *in vitro* to TNP-modified syngeneic spleen cells and that the effector T cells were directed against these "new antigenic determinants" (7). The targets could be TNP-modified normal spleen cells or TNP-modified P815 mastocytoma cells providing that the stimulator and target cells were from mice of the same strain. Thus, the T-cell response was directed to TNP-modified cell-surface proteins and, as would now be obvious to first-year immunology students, the response was H2 restricted.

Adoptive transfer of thymus-derived lymphocytes from mice rendered tolerant to BSA restored the ability of thymectomized-irradiated recipient mice to produce antibody to BSA after immunization with SA-conjugated BSA (8). This experiment demonstrated that tolerized T cells could respond to "new antigenic determinants" resulting from the chemical modification.

Table 1
Chemical Conjugation of Commonly Used Haptens

<i>Hapten</i>	<i>Mode of Linkage</i>			<i>Entities Modified</i>
	<i>Direct</i>	<i>Diazonium</i>	<i>S-mustard</i>	
Dinitrophenyl (DNP)	+			ϵ -amino acid groups (lysine, leucine)
Trinitrophenyl (TNP)		+	+	tyrosine, histidine tyrosine, histidine
Arsanilic acid				
Sulfanilic acid (SA)		+		tyrosine, histidine
Phosphorylcholine		+		tyrosine, histidine
AED ^a	+			sulphydryl groups

^a*N*-iodoacetyl-*N'*-(5-sulfonic-1-naphthyl) ethylene diamine

Finally, the autoreactivity induced by hapten-modified immunogens applied to T-cell-mediated as well as antibody responses. Immunization of mice with TNP-conjugated syngeneic spleen cells resulted in the development of DTH to unmodified syngeneic lymphoblasts (9). This phenomenon was H2 restricted. The stimulating moiety was contained within a low-molecular-weight fragment of the H2 heavy chain that was extracted from haptenized cells but did not contain the hapten—further evidence for the importance of the “new antigenic determinant.”

4. CHEMICAL CLASSIFICATION OF HAPTENS

The number of synthetic compounds that can function as haptens is limited only by the imagination of the organic chemist. However, most of the experiments published over the past 25 yr have utilized the six haptens listed in Table 1. The reason for focusing on such a small sampling of haptens is that the immunological responses appear to depend less on the structure of the hapten than on the chemistry of its conjugation to protein.

Two of the most intensely studied haptens, DNP and TNP, are attached to proteins by nucleophilic substitution; apparently, “the DNP-NH bond in proteins is even more stable than the peptide bond” (10). DNP and TNP induce cross-reacting antibody responses and the effect of these nitrophenyls on carrier proteins (whether soluble or bound to cells) is essentially identical. The covalent bond is critical, since treatment with TNP stearoyl dextran, which binds by noncovalent forces, does not result in haptic modification (11). Other haptens, such as SA, must be introduced into proteins by a diazonium reaction; i.e., a diazonium salt is made by treatment with sodium nitrate (12). Hanna et al. (13) demonstrated differences in the antibody and delayed-type hypersensitivity (DTH) responses induced in guinea pigs by DNP-modified BSA depending on the method used to prepare the conjugate: directly conjugated DNP induced higher antibody titers to DNP whereas the DNP-azo-BSA induced higher titers to native BSA. Both conjugates induced strong DTH responses to both modified and native BSA.

These subtle differences may be explained by the tendency of DNP (and TNP) to couple to the hydrophilic portions of membrane molecules that are rich in lysine and

leucine, whereas the diazonium conjugates have an affinity for tyrosine and histidine. A consequence of this differential chemistry is that conjugation with the hapten, *N*-iodoacetyl-*N'*-(5-sulfonic-1-naphthyl) ethylene diamine (AED), which binds to sulphydryl groups, does not interfere with subsequent modification of the same protein with TNP (14).

5. MOLECULAR IMMUNOLOGY OF HAPten MODIFICATION

What are the “new antigenic determinants” produced by hapten conjugation that must have excited and perplexed the early investigators of hapten immunology? An impressive body of work by H. U. Weltzien’s group appears to have solved the mystery. By immunizing mice to TNP-modified syngeneic spleen cells, they obtained cytotoxic T cells (CTLs) that lysed these cells in an H2-restricted manner. The CTLs also recognized TNP-modified peptides extracted from tryptic digests of TNP-modified BSA (15). Subsequently, these investigators synthesized a variety of class I major histocompatibility complex (MHC)-binding peptides that contained TNP-lysine at various positions. CTL clones were tested for ability to lyse target cells that had bound one of the synthetic peptides (16).

They demonstrated the following: T cells recognized the MHC-associated TNP-modified peptides whereas hapten-modification of the MHC complex itself was of minimal significance (17); immunization of mice with a TNP-modified peptide sensitized the animals for a positive contact sensitivity reaction upon topical application of trinitrochlorobenzene (TNCB); the vast majority of CTL clones responded to multiple H2-binding peptides that had in common a TNP-lysine in position 4, whereas recognition was largely independent of the amino acid sequence (16); a minor fraction of TNP-specific T-cell clones recognized only certain sequences of TNP-modified peptides. The designer peptides revealed a complex antigenic determinant composed of TNP-lysine in position 7 and unmodified amino acids in positions 3 and 4 (16). Interestingly, these T-cell clones also recognized *unmodified* peptides; i.e., there was associative recognition of *unmodified* peptides by T-cell clones generated by immunization with hapten-modified peptides.

The authors explained these results by postulating self-reactive T cells that survive thymic selection because they have low affinity for self-peptides. Haptenization then would increase the binding of T-cell receptors (TCRs) to self peptide enough for T-cell activation, and, once activated, the T cells could react with unmodified peptide.

This work was performed with peptides that stimulated CD8+ cells through the class I MHC pathway. However, similar findings have been reported with TNP-modified peptides presented through the class II pathway to CD4+ T cells (18). Moreover, these phenomena are not limited to peptides modified with TNP: T cells reactive to an otherwise nonimmunogenic peptide derived from hen egg lysozyme (HEL) could be obtained from mice after immunization with HEL that had been modified with the hapten, phosphorylcholine, which conjugates protein through a diazonium linkage (19).

6. MODELS OF HAPten-INDUCED AUTOIMMUNITY

As described above, in his studies on cross-reactivity of hapten-modified and native proteins, Weigle (3) serendipitously found that mice immunized with hapten-modified thyroglobulin developed histological evidence of autoimmune thyroiditis. More recently

haptens have been intentionally applied to develop animal models of what are presumed to be human autoimmune diseases. Neurath et al. (20) applied small amounts of the hapten, trinitrobenzene sulfonic acid (TNBS) (a derivative of TNP), to the rectal mucosa of mice. This resulted in the development of a chronic transmural colitis accompanied by diarrhea and weight loss that mimicked human Crohn's disease. Histologic examination showed abundant CD4+ T cells that produced mRNA for gamma interferon *in situ*. Of great interest was the observation that the colitis persisted for at least 2 mo, obviously long after all hapten-modified cells had been shed and cleared. Therefore, the T cells, initially induced by hapten-modified mucosa, were able to recognize and react against unidentified proteins on normal mucosa.

Similarly, Rennke et al. (21) induced acute nephritis by infusion into the rat kidney of azobenzeneearsonate, a hapten that conjugates protein through a diazonium linkage. They observed massive destruction of the renal cortex accompanied by infiltration with mono-nuclear leukocytes. No nephritis was observed in the uninjected kidney, but the experiment was terminated after 5 d—not enough time for an immune response against unmodified kidney cells to have developed.

6.1. Drug-Induced Autoimmunity

Ingested drugs can act as haptens, which combine with normal tissue proteins forming immunogenic complexes that are recognized by T cells. Although drugs tend not to be highly chemically reactive molecules, their metabolites may well be. An example is penicillin, which can be associated with autoimmune hemolytic anemia. Although not likely to conjugate proteins itself, in solution penicillin spontaneously forms penicillic acids with a highly reactive oxazolone group (22) that modifies proteins in the membranes of erythrocytes.

A frequently studied example of drug-induced autoimmunity is drug-induced hepatitis that can be associated with the commonly used antihistamine, chlorpheniramine (23). Peripheral blood lymphocytes (PBLs), and several CD4+ clones derived from them, proliferated upon stimulation with a mixture of chlorpheniramine and a liver-derived protein, but not to the drug alone. These results suggested that the T-cell response was directed against the protein that had been modified, in a hapten-like manner, by the drug. In this system there was no detectable T-cell response to liver-derived protein alone, and the hepatitis resolved as soon as administration of the drug was stopped.

There is evidence that the immune response induced by protein-drug conjugates can lead to autoimmunity against the native proteins. Drug-induced (e.g., hydralazine, quinidine, or isoniazid) systemic lupus erythematosus is associated with the development of auto-antibodies, particularly antinuclear antibody (24). As a result, the manifestations of the disease can continue even after withdrawal of the offending drug.

7. IMMUNOTHERAPY OF EXPERIMENTAL TUMORS WITH HAPten-MODIFIED VACCINES

There is considerable evidence that the failure of immunotherapy to eradicate cancers, whether spontaneous human cancers or experimental transplantable tumors, is a result of immunological tolerance. The most striking illustration of this hypothesis remains the work of Mullen et al. (25). They produced two variants of the fibrosarcoma 1591—a regressor tumor that was highly immunogenic and always rejected by normal mice and

a progressor tumor that had become nonimmunogenic as a result of losing a tumor-rejection antigen. Surprisingly, animals that had been implanted with the progressor tumor were unable to reject even a small challenge of the regressor tumor. These progressor tumor-bearing mice were not generally immunosuppressed, since they generated normal immune response to alloantigens, allogeneic tumors, and certain other syngeneic tumors. Their immunological tolerance was tumor-specific.

However, it was possible to break tolerance against the progressor tumor by haptenization. Thus, mice immunized with TNP-modified regressor tumor rejected a challenge with TNP-modified progressor tumor. Moreover, 28 d later they were able to reject a challenge with *unmodified progressor tumor* (26). The protection against the progressor tumor also could be transferred by splenic T cells obtained from TNP-regressor-immunized mice.

Before and after this work was published, a number of other investigators demonstrated that modification of tumor cells with DNP or TNP increased the efficacy of vaccines. Actually, this idea seems to have been first proposed by Mitchison (27), who in 1970 discussed a number of ways of inducing “helper determinants” onto tumor cells. For example, Cavallo and Forni (28) found that mice immunized with DNP-modified mammary adenocarcinoma cells exhibited delayed tumor appearance and slower tumor growth after challenge with unmodified tumor cells. Galili et al. (29) performed a similar experiment with a virally induced lymphoma with more impressive results: Immunization with TNP-modified tumor appeared to be effective in increasing the percentage of long-term survivors even in animals in whom the unmodified tumor was nonimmunogenic. Roth et al. (30) were able to demonstrate protective immunity in guinea pigs immunized with a DNP-modified chemically induced sarcoma. Of course, this approach did not work with all tumors (31), but published reports showed consistently positive results.

Fujiwara et al. (32) took a somewhat different approach. They found that a fairly large (8-mm diameter) plasmacytoma could be made to regress completely by intratumoral injection of TNCB, provided that the mice had been previously sensitized by topical application of TNCB. As a result, these animals became resistant to challenge with unmodified tumor cells. This immunotherapy was particularly interesting because it worked not only with a transplantable tumor, but also with an autochthonous tumor that was induced in the mice by inoculation of methylcholanthrene.

The most recent publication on the use of hapten modification for experimental immunotherapy is from Sojka et al. (33). They used the highly metastatic 410.4 tumor that had originated from a spontaneous murine mammary carcinoma. The tumor was injected into the mammary fat pad and was allowed to grow to 6- to 8-mm diameter and then excised. Following surgery mice were treated with multiple injections of a vaccine consisting of irradiated tumor cells haptenized with DNP and then mixed with BCG. Low-dose cyclophosphamide was administered 3 d prior to each vaccine injection (34,35). Control mice received the identical treatment regimen except that the tumor cells in the vaccine were irradiated but *not hapten modified*. These experimental conditions were designed to mimic the postsurgical adjuvant protocols frequently used in clinical vaccine studies and, specifically, to experimentally reproduce our observations in melanoma patients, which are described below.

The result was positive and highly reproducible: mice that received DNP-modified vaccine had significantly longer relapse-free survival than animals receiving the unmodi-

fied vaccine, which, incidentally, was no better than saline. The protective effect of the haptenized vaccine was dependent on both CD4+ and CD8+ T cells. Moreover, both gamma interferon and tumor necrosis factor were essential, since in vivo depletion of either with a monoclonal antibody abrogated the protective effect.

8. HUMAN AUTOLOGOUS, HAPTEN-MODIFIED VACCINE

8.1. Rationale

Our clinical studies, which began in 1988, were stimulated by the promising results that had been reported in animal models up to that time and by the seminal observations on hapten immunology made over the prior 50 yr that have been described above. The scientific underpinning of the project was strengthened by Weltzien's work on hapten-modified peptides, which appeared shortly after our trials began. We reasoned that the failure of human cancer vaccines to immunize cancer-bearing patients was due to immunological tolerance to one or many rejection antigens. Since haptenization could break tolerance even to normal cells, it might be able to make tumor antigens immunogenic enough to see clinical effects.

All of our work has been performed with an autologous vaccine because the response to hapten-modified proteins is MHC dependent and because of the strong rationale for the use of autologous cells that has been developed and extensively discussed by others (36–39). In addition, all of our protocols have incorporated low-dose cyclophosphamide to take advantage of its ability to potentiate cell-mediated immune responses. This strategy is supported by hundreds of published observations (34,35,40,41) and will not be discussed further here.

Finally, we chose DNP as the hapten because of its long record of clinical use without significant toxicity. Most normal subjects and the majority of cancer-bearing patients can be sensitized by topical application of dinitrochlorobenzene (DNCB) (42), and their PBLs proliferate in response to DNP-modified autologous lymphocytes (43). Moreover, DNP is easy to conjugate to tumor cells, since it does not require chemical modification.

All of the studies described here were approved by the Institutional Review Board of Thomas Jefferson University, and informed consent was obtained from all patients.

8.2. Preparation and Administration of the Vaccine

Metastatic tumor is excised, maintained at 4°C, and delivered to the laboratory within 48 h of excision. Tumor cells are extracted by enzymatic dissociation with collagenase, aliquotted, frozen in a controlled-rate freezer, and stored in liquid nitrogen in a medium containing human albumin and 10% dimethylsulfoxide until needed. On the day that a patient is to be treated, an aliquot of cells is thawed, washed, and irradiated to 2500 cGy. Then the cells are washed again and modified with DNP by the method of Miller and Claman (44). This involves a 30-min incubation of tumor cells with DNFB, followed by washing with saline. After washing, the cells are counted, suspended in 0.2-mL Hanks solution with human albumin, and maintained at 4°C until administered.

Just prior to injection, 0.1 mL of Tice BCG (Organon Teknika Corp., Durham, NC) is added to the vaccine. Then the mixture is drawn up in a 1-mL syringe and injected intradermally, usually into the upper arm. Although most of the animal tumor models with haptenized vaccines did not include an immunological adjuvant, it was clear from the work of Weigle (3) that admixture of an adjuvant with haptenized thyroglobulin

Table 2
Summary of Vaccine Schedules Tested

Schedule	No. Doses Cyclophosphamide	DNFB Sensitization	Vaccine Schedule	Vaccine Dosage Range	BCG mixed with:
A	3	+	q 28 days × 8	5.0–25.0 × 10 ⁶	every dose
B	3	+	weekly × 12	5.0–25.0 × 10 ⁶	1/3 of doses
C	3	+	weekly × 12	5.0–25.0 × 10 ⁶	every dose
D	1	–	weekly × 6	2.5–7.5 × 10 ⁶	every dose
E	1	–	weekly × 6	0.5–2.0 × 10 ⁶	every dose

Notes. Schedule A: Cyclophosphamide 300 mg/M² iv 3 d before DNFB and 3 d before first two doses of vaccine.

Schedule B: Cyclophosphamide 300 mg/M² iv 3 d before DNFB and 3 d before each 6-wk series of vaccine; half of the vaccines were DNP modified and half unmodified.

Schedule C: Cyclophosphamide 300 mg/M² iv 3 d before DNFB and 3 d before each 6-wk series of vaccine; all vaccines were DNP modified.

Schedules D, E: Cyclophosphamide 300 mg/M² iv 3 d before first vaccine only; booster injections of vaccine were given at 6 and 12 mo.

augmented the development of the antithyroid response. We chose BCG because it is a potent adjuvant and an approved drug as well. To minimize the local reaction to BCG, the dose was progressively attenuated 10-fold through a series of vaccine administrations. The initial BCG dose was a 1:10 dilution (1–8 × 10⁶ CFU) and subsequent BCG doses were a 1:100 dilution (1–8 × 10⁵ CFU) and a 1:1000 dilution (1–8 × 10⁴ CFU).

8.3. Dose and Schedule of Administration

Five vaccine dosage schedules have been tested and are summarized in Table 2. The animal tumor models, particularly the paper of Fujiwara et al. (32), prompted us to presensitize patients with DNFB by topical application of a 1% solution in acetone-corn oil, and this was done in schedules A, B, and C. Subsequently, the presensitization was found to be unnecessary (but not deleterious) for the induction of maximum DTH to autologous melanoma cells and it was omitted for schedules D and E.

For schedule A, DNP-vaccine mixed with BCG was administered every 4 wk for a total of eight doses; cyclophosphamide 300 mg/M² was administered 3 d before the first and second doses. All vaccine injections were given in the same site on a limb (usually the upper dorsal arm) that had not been subjected to a lymph node dissection. For schedule B, vaccine was administered weekly for 6 wk; after a 4-wk reevaluation period vaccine was again administered weekly for 6 wk. The first three vaccines of each course were DNP modified and the last three were unmodified. BCG was admixed only with the first and fourth vaccine of each course. All of the DNP-vaccine injections were given into one area, and all of the unmodified vaccine injections were given into a second area. Cyclophosphamide 300 mg/M² was administered 3 d prior to the start of each vaccine course. Schedule C was identical to schedule B, except that all vaccines were DNP modified and all were mixed with BCG. Schedules D and E were simplified regimens in which DNFB presensitization was omitted and only one series of six weekly DNP-modified vaccines was administered; these schedules differed only by dose ranges: D = 2.5 – 7.5 × 10⁶ vs E = 0.5 – 2.0 × 10⁶ intact tumor cells per dose.

Table 3
Summary of Patients Treated With DNP-Modified Autologous Vaccine

Melanoma:	1) Measurable metastases 2) Postsurgical adjuvant, stage III 3) Postsurgical adjuvant, stage IV 4) Postsurgical adjuvant, low dose	97 ^a 214 ^a 20 ^b 76 ^b
	Total	407
Ovarian Carcinoma:		30^b
Renal Cell Carcinoma:		10^b
Total:		447

^aClinical and immunological results discussed in text.

^bOnly immunological results discussed in text.

8.4. Description of Patients

Table 3 provides a summary of the patient populations treated with the DNP-modified autologous vaccine from October 1988 through April 2001. The observations of immunological and clinical responses described below were performed in the melanoma subjects. The results obtained in ovarian and renal cell carcinoma will be presented in a separate section.

8.5. Toxicity of DNP-Modified Autologous Vaccine

In most patients, the toxicity was limited to the reaction at the vaccine injection site. All patients developed pruritic papules that progressed to pustules, sometimes with small ulcerations, that resolved into small white or pink scars. The intensity of the local reactions was ameliorated by reducing the dose of bacille Calmette-Guérin (BCG). As expected, about one-third of patients developed nausea, sometimes with vomiting, following administration of cyclophosphamide, but systemic toxicity caused by the vaccine was uncommon: Less than 5% of patients noted fever or chills following vaccine administration, and no patient experienced a decrease in performance status. One patient developed generalized urticaria 15 min after injection of her fourth dose of DNP-vaccine; this was treated with an antihistamine and resolved in 5 d without sequelae. Three patients developed erythema around their lymphadenectomy sites following vaccine administration, which was asymptomatic and spontaneously abated. Skin biopsy of one of these patients showed a nonspecific vasculitis. There were no significant changes in blood counts or routine serum chemistries. We observed no clinical evidence of autoimmunity. Specifically, no patients developed vitiligo following treatment.

8.6. Development of Delayed-Type Hypersensitivity Responses

8.6.1. METHOD FOR DTH TESTING

Patients were tested for DTH by modification of standard methodology (45). Cryopreserved melanoma cell suspensions and PBLs were thawed, washed, and irradiated (2500 cGy). DNP modification of melanoma cells and PBLs was performed as

described above. Melanoma cells (1×10^6) and PBLs (3×10^6), each either DNP modified or unmodified, were suspended in Hanks balanced salt solution without serum, phenol red, or antibiotics and injected intradermally into the ventral forearm. The mean diameter of induration was measured after 48 h. A positive response was defined as: maximum diameter of induration ≥ 5 mm. Patients were also skin tested with intermediate strength purified protein derivative (PPD)(5 TU). DTH testing was performed before the treatment program was initiated and at various times post-treatment. Analyses were performed by determining the maximum DTH response exhibited by each patient to each of the test reagents.

Pretreatment positive DTH responses to autologous melanoma cells, either DNP modified or unmodified, were observed in 8% of patients and were generally small (median = 6 mm, range = 5–18 mm).

Most patients were tested for DTH to autologous melanoma cells that had been dissociated with enzymes (collagenase and DNase) and to melanoma cells that had been mechanically dissociated only. There was a strong correlation between DTH responses to the two preparations (adjusted squared multiple R = 0.752). Twenty-six patients (9%) developed an apparent DTH response to the enzymes, as measured by skin testing with enzyme-coated autologous PBLs. Of these, 16 had been treated on Schedule B, which includes immunization with unmodified as well as DNP-modified melanoma cells. If a patient developed DTH to enzyme-coated PBLs, we analyzed only their DTH response to mechanically dissociated melanoma cells.

8.6.2. RESULTS OF DTH TESTING

A summary of the DTH responses observed following administration of DNP-vaccine is shown in Table 4. The responses of patients with measurable metastases (Group 1 in Table 3) were similar to those who had undergone resection of clinically evident disease (Groups 2–4 in Table 3). Almost all patients developed positive responses to DNP-modified autologous melanoma cells and to PPD; these responses were usually at least 10 mm diameter. Responses to unmodified autologous tumor cells were induced only in a subset of these patients and were smaller (usually 5–10 mm diameter).

Following vaccine treatment, five patients (2%) exhibited a small (5–6 mm) DTH response to autologous, unmodified PBLs after treatment. This could have represented a T-cell response against normal-tissue antigens, but is more likely to be artifactual, since no other manifestations of autoimmunity were observed. As expected, 52% of patients developed DTH to DNP-modified PBLs, but in no case was the response cross-reactive with *TNP*-modified PBLs.

These results may be interpreted as follows: (a) Modification of melanoma cells with DNP produces “new antigenic determinants” that induce strong and consistent cell-mediated immune responses, even in cancer-bearing subjects. (b) Evidently, these responses are sometimes associated with cell-mediated immunity against one or more determinants on unmodified melanoma cells. Such determinants appear to be melanoma associated since they are not present on normal lymphocytes. This interpretation is consistent with the large body of work on hapten responses in experimental systems that preceded it, particularly the work of Weltzien et al. (16). Their observation of a response to hapten-modified peptides dominated by T-cell clones that are sequence independent parallels our observation with DTH to DNP-modified tumor cells. Their finding of a

Table 4
Delayed-Type Hypersensitivity Responses Following DNP-Vaccine Administration

Patient Population	Test Reagent	DTH (mm) Median (range)	% Patients with Response ≥5 mm	% Patients with Response ≥10 mm
Postsurgical Adjuvant	DNP-Modified TC	20 (4–70)	99%	87%
	Unmodified TC	5 (0–22)	56%	15%
	PPD	25 (8–85)	100%	99%
Advanced Metastases	DNP-Modified TC	17 (0–70)	95%	77%
	Unmodified TC	5 (0–22)	57%	13%
	PPD	24 (0–85)	99%	98%

Note. DTH: largest diameter of induration measured at 48 h. TC: autologous melanoma cells. PPD: Purified protein derivative of *Mycobacterium tuberculosis*.

much smaller number of T-cell clones that are sequence dependent and reactive with unmodified peptides predicts our observations on DTH to unmodified tumor cells.

8.7. Corresponding In Vitro T-Cell Response to DNP-Modified Cells

The agreement between our results and the animal studies is strengthened by experiments that we performed with T cells obtained from DNP-vaccine-treated subjects. PBL obtained from 8 of 11 patients proliferated, and in 5 of 11 produced gamma interferon, upon stimulation with DNP-modified autologous lymphocytes or melanoma cells. The response was human leukocyte antigen (HLA) restricted and hapten restricted, since no responses were elicited by unmatched DNP-modified allogeneic cells or by TNP-modified autologous cells. Finally, a CD8+ cell line obtained from one of these responding PBLs killed DNP-modified autologous melanoma cells but was not cytotoxic for DNP-modified allogeneic tumor cells (46).

Furthermore, we were able to identify MHC-associated, DNP-modified peptides that reproduced these responses (47). Peptides, extracted from DNP-modified melanoma cells by an acid elution technique and fractionated by high-performance liquid chromatography (HPLC), were loaded onto autologous B lymphoblasts, which were tested as stimulators to the cell line described above. Unexpectedly the stimulatory capacity of peptides was confined to a single HPLC fraction; spectrophotometric analysis of this fraction showed that the peptides were conjugated to DNP. Perhaps this peak represented a family of peptides corresponding with Weltzien's dominant peptides with the hapten-modified lysine in position 4.

8.8. Clinical Importance of DTH Responses

An important observation that has been sustained over the course of these studies is the association of a positive DTH response to *unmodified*, autologous melanoma with prolonged survival. Thus, in the measurable metastases group, the survival of patients who developed a positive DTH to unmodified tumor cells was significantly longer than the survival of those who did not: 16.5 mo vs 8.4 mo, respectively ($p = 0.023$, log-rank test) (48). In the postsurgical adjuvant group, the development of a positive response to unmodified tumor cells was associated with significantly greater 5-yr survival ($p < 0.001$,

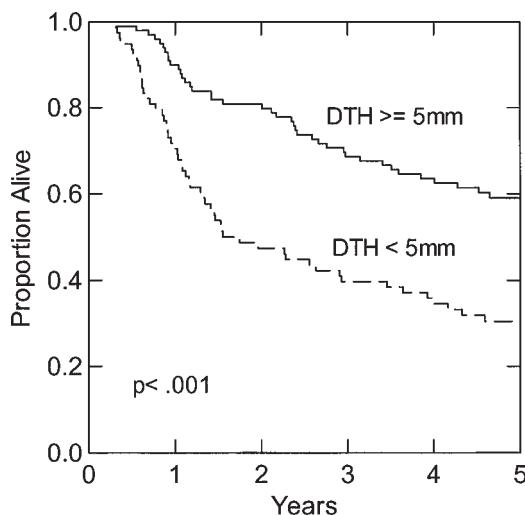


Fig. 1. Effect of DTH to unmodified autologous melanoma cells on overall survival. Patients had clinical stage III melanoma and were disease-free following lymphadenectomy (Group 2 in Table 3) (49). The overall survival of patients who developed DTH ≥ 5 mm induration (solid line, $N = 99$) is compared with that of patients whose maximum DTH response was <5 mm (dashed line, $N = 78$). Difference between the curves: $p < 0.001$, log-rank test.

log-rank test) (Fig. 1). This effect remained significant in a multivariate analysis that included important clinical prognostic variables, such as the number of positive lymph nodes (49). In contrast, the magnitude of DTH response to DNP-modified autologous melanoma cells had no significant impact on survival.

8.9. T-Cell Responses at the Tumor Site

8.9.1. TUMOR INFLAMMATORY RESPONSE

A rather surprising observation was made early into the first clinical trials of DNP-modified autologous vaccine: the development of inflammatory responses in metastatic sites (50). These responses were initially observed in superficial (nodal or subcutaneous) metastases, and consisted of marked erythema, warmth, and tenderness of the tumors and the overlying skin. Responding metastatic lesions varied in size from 5-mm diameter skin metastases to 10-cm lymph node masses. The number of inflamed tumors on a single patient ranged from 1 to >100 . In some patients who had multiple superficial metastases, the inflammatory response involved all of the observable lesions, whereas others had inflammation in 25–75% of their visible tumors. The time from the beginning of vaccine treatment to an observable inflammatory response was fairly long—2–4 mo.

Biopsy of superficial metastases excised following treatment with DNP-vaccine showed a striking histologic change: the tumors had become infiltrated with T lymphocytes (51). Such infiltration is not observed in subcutaneous metastases obtained from patients prior to immunotherapy; only occasionally were significant numbers of lymphocytes identified, and they were usually limited to perivascular areas. The histology is illustrated in Fig. 2.

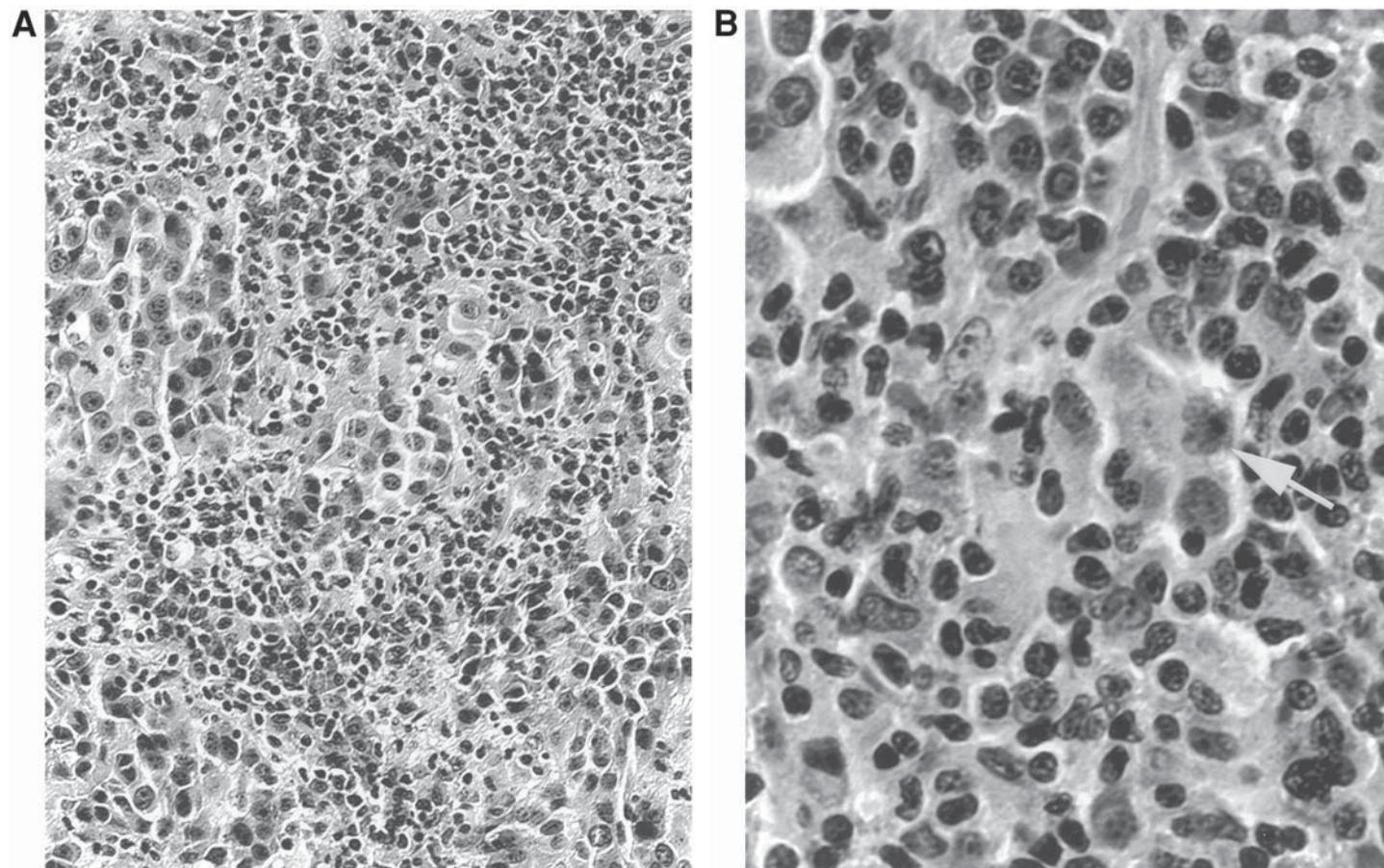


Fig. 2. Photomicrograph showing inflammatory response in subcutaneous metastasis. The clinically inflamed tumor was excised 5 mo after beginning treatment with DNP-vaccine. **(A)** ($\times 200$) The tumor tissue is intensely infiltrated with lymphocytes. **(B)** ($\times 600$) Necrosis of individual tumor cells is apparent (arrow). (Reprinted with permission from 48.)

We quantified the inflammatory responses by flow cytometric analysis of T lymphocytes in cell suspensions made from subcutaneous metastases excised before and after DNP-vaccine treatment. The median number of T cells in 15 post-vaccine tumors was 41%, with some tumors containing more than 50% T cells. In contrast, in subcutaneous metastases excised without prior immunotherapy T cells were sparse (median = 9%), and significantly less frequent than in post-DNP-vaccine-treated tumors. Lymphocytes other than T cells were rare; the mean proportion of CD19(+) B cells and CD56(+) natural killer cells in tumor suspensions was 1% and 4%, respectively. T cells were predominantly CD8(+); the mean CD8/CD4 ratio was 5.0 vs 1.0 in matched PBLs.

A high proportion of infiltrating T cells expressed HLA-DR, suggesting that they were activated (52). Expression of DR was not accompanied by increased cell-surface expression of the interleukin-2 (IL-2) receptor, CD25. However, infiltrating T cells strongly expressed CD69, an early marker of T-cell activation (53).

The production of cytokines by lymphocytes infiltrating the metastases was studied by analyzing the tissues by a standard RT-PCR (polymerase chain reaction) technique (54). Post-vaccine, inflamed biopsies contained messenger RNA for γ interferon (five of eight), IL-4 (four of eight) or both (three of eight), and for tumor necrosis factor (TNF) (four of seven). In contrast, γ interferon mRNA was detected in only one of 17 and TNF mRNA in two of 16 control specimens (pretreatment lymph node metastases or noninflamed subcutaneous metastases). Since γ interferon and TNF are critical cytokines in the initiation and perpetuation of cell-mediated immune responses, their presence within these tumors has biological significance.

8.9.2. CLONAL EXPANSION OF T CELLS AT THE TUMOR SITE

We investigated the rearrangement of the TCR β chain (TCR-V β) as well as the junctional diversity in T cells infiltrating melanoma metastases following treatment with DNP-vaccine (55,56). In 19 of 26 control specimens, V-D-J length analysis showed the expected polyclonal patterns. In contrast, post-vaccine tumors from 9 of 10 patients showed dominant peaks of V-D-J junction size in one or more V β families. Dominant peaks were seen most frequently in six V β families (V β 7, 12, 13, 14, 16, 23) and were never seen in seven others. Further analysis of the oligoclonal V β products showed dominant peaks in the J-region as well. Of particular interest was the finding that V β and J β peaks were similar in inflamed metastases obtained at different times or from different sites from the same patient. An example of the V-D-J size distribution histograms for one patient (20297) is shown in Figure 3. The dominant peaks seen in the post-vaccine tumors do not appear to be present in the matched PBLs.

Finally, the amplified PCR products from seven of these specimens were cloned and sequenced and the amino acid sequence of the CDR3 deduced. In six of seven specimens the same CDR3 sequence was repeated in at least two clones and, in five of seven in at least three clones. That these novel clones are functional was shown by isolating T-cell lines from two infiltrated skin metastases by enrichment for TCR-V β 14 T cells. This line displayed HLA-class I-restricted lysis of the autologous melanoma cells (55).

These results indicate that vaccination with autologous, DNP-modified melanoma cells can expand selected clones of T cells that migrate to the tumor site that were not detectable prior to immunization. Moreover, such clones are potentially destructive to the tumor. This is an observation that has not been made with other human tumor vaccines.

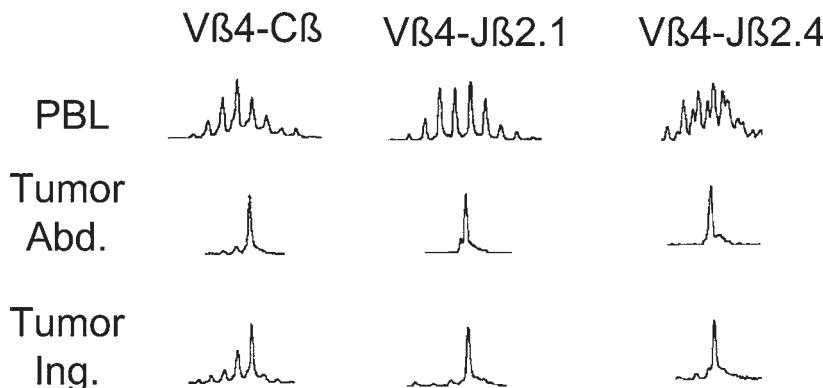


Fig. 3. Examples of histograms of V-D-J junction size distributions from two inflamed metastases excised from one patient. Amplification with $V\beta$ primers revealed a clonal peak in $V\beta$ -4 for both abdominal (Abd) and inguinal (Ing) metastases, both of which became inflamed after treatment of the patient with DNP-vaccine, but a polyclonal distribution in matched PBLs. Amplification with $J\beta$ primers showed clonal peaks in $J\beta$ -2.1 and 2.4 for both tumors but not in PBLs. (Reprinted with permission from 56).

8.10. Clinical Effects of DNP-Modified Autologous Vaccine

8.10.1. PATIENTS WITH MEASURABLE METASTASES

We have reported the results of a series of clinical trials of patients with surgically incurable metastatic melanoma with measurable metastases (48); there were 97 patients, of whom 83 were evaluable (Group 1 in Table 3). Among the 83 evaluable patients there were 11 responses—2 complete, 4 partial, and 5 mixed; 2 patients were judged to have stable disease. Both complete responses and two of the four partial responses occurred in patients with lung metastases. Response durations were as follows: partial responses—5, 6, 8, and 47+ mo; complete responses—12, 29 mo. Two examples of antitumor responses are provided below:

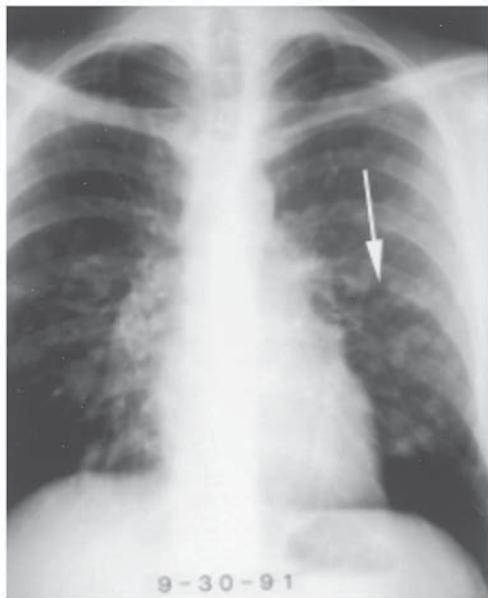
Patient no. 20063, a 28-yr-old man, developed multiple bilateral lung metastases shortly after tumor tissue had been obtained from a regional lymph node metastasis (Fig. 4, first panel). These metastases increased in size and number just prior to starting DNP-vaccine treatment (Fig. 4, second panel). After a course of DNP-vaccine administration, the appearance of the metastatic nodules was unchanged (not shown). However, 2 mo later, the metastases had completely regressed (Fig. 4, third panel). The patient remained tumor-free until 1 yr later when mediastinal and hilar recurrence was noted. His survival from beginning of DNP-vaccine treatment was 34.5 mo.

Patient no. 20254, a 77-yr-old man, presented simultaneously with a regional lymph node metastasis in the neck and 2-cm diameter mass in the lung adjacent to the cardiac border that increased in size over 2 mo observation. Five months after beginning DNP-vaccine treatment, the same mass was thought to be slightly smaller. The mass continued to slowly regress and by the 2-yr point it had regressed completely. After a remission of 29 mo, the patient developed recurrent melanoma in the anterior mediastinum, which was incompletely resected. His overall survival was 48.4 mo.

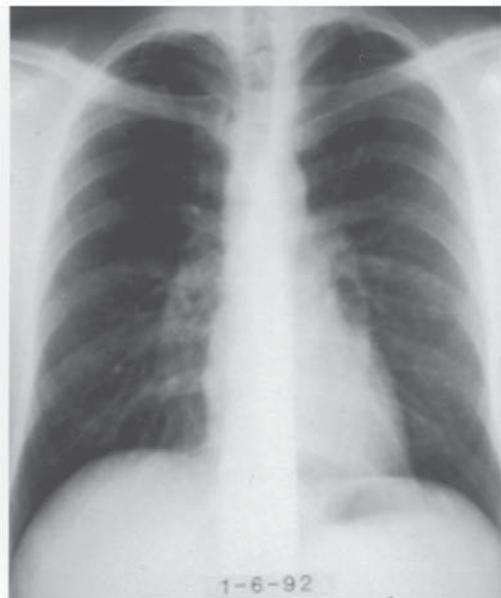
These examples of regression of lung metastases are noteworthy because the regressions developed slowly and only after a latent period. Evidence of response required at



**July, 1991
pre-vaccine**



**September, 1991
vaccine begun**



**January, 1992
4 months post-vaccine**

Fig. 4. Regression of lung metastases in patient no. 20063 after administration of DNP-modified, autologous vaccine. Multiple lung metastases are seen in July 1991, most prominently in the left lower lobe, which increased in size and number in the September 1991 X-ray. Four months after beginning DNP-vaccine treatment (January 1992), the metastases had completely regressed. (Reprinted with permission from 48.)

least 4–5 mo, and in two of these cases maximum regression required at least 1 yr. Patients who had an antitumor response (complete, partial, or mixed) had a significantly longer survival time—about 2.5 times that of nonresponders ($p < 0.01$, log-rank test and by multivariate analysis).

8.10.2. POSTSURGICAL ADJUVANT PATIENTS

We hypothesized that the ability of autologous DNP-vaccine to produce antitumor effects was limited by the large tumor burden of patients with clinically evident metastases. Therefore, it seemed reasonable to test the vaccine in a setting in which the tumor burden was much lower. Patients with bulky but resectable regional lymph node metastases constitute an ideal group, since the metastatic masses provide a source of cells for preparing vaccine but the postsurgical tumor burden is below the level of clinical detection.

Therefore, we conducted a series of trials in this patient population. All of the study subjects had regional lymph node metastases with a palpable mass of at least 3 cm diameter. They were treated by standard lymphadenectomy, and tumor cells were extracted and cryopreserved from the nodal tissue. To be eligible, the patients had to be tumor-free by postoperative evaluation, and the vaccine treatments had to be started within a month of surgery.

The initial results of these studies have been reported (49), and the studies completed accrual in 1998 with a total of 214 (Group 2 in Table 3). Twenty of these patients had in-transit metastases as well, and 40 had clinically evident metastases to two nodal sites. With a median follow-up time of 5.1 yr (2.5–10.9 yr) the 5-yr overall survival rate is 46% (one nodal site = 48%, nodes plus in-transit metastases = 50%, two nodal sites = 36%). As expected, extent of nodal involvement was a strong predictor of 5-yr overall survival (mass only = 65%, mass plus one or two microscopically [+] nodes = 43%, mass plus three or more microscopically [+] nodes = 25%).

The patients receiving DNP-vaccine appear to have relapse-free and overall survivals that are higher than have been reported with surgery alone (57,58). Moreover, the survival percentages appear to be at least comparable to those of patients treated with high-dose interferon (59,60), and were achieved with minimal toxicity.

8.11. Importance of Dosage Schedule

In planning these clinical trials, the animal models were not useful in choosing a dosage schedule for the DNP-modified vaccine. Almost all models employed a single dose of vaccine and rarely were single doses compared with multiple doses. However, from the outset, we reasoned that multiple injections of vaccine might lead to better T-cell responses and were unlikely to be disadvantageous. The number of tumor cells per dose was extrapolated from murine systems in which 10^6 – 10^7 cells is the rule.

Our demonstration that the development of DTH to unmodified melanoma cells was critical to its clinical effectiveness has allowed us to study the dose response of the vaccine using DTH as the response. An analysis of 284 patients (Groups 2–4 in Table 3) who were treated following resection of regional or distant metastases showed no significant association between the magnitude of DTH and the number of live (trypan blue-excluding) melanoma cells administered per dose over a dosage range of 0.5 – 25.0×10^6

(61). Surprisingly, there was a small but significant positive relationship between the mean number of dead cells in the vaccines of a given patient and that patient's maximum DTH response, but that parameter accounted for <5% of the variability.

In contrast, we have found that the schedule of vaccine administration is critically important to its effectiveness. Of the five schedules listed in Table 2, the DTH responses induced by schedules B and C were significantly inferior to those induced by schedules A, D, and E ($p < 0.001$, Kruskal Wallis test). This result was not due to inadvertent patient selection since all patients were clinically tumor-free and the schedules did not affect the magnitude of the DTH responses to PPD.

It has become apparent that the variation in intensity of DTH responses to autologous melanoma cells among patients receiving different treatment schedules was likely to be explained by the timing of what we have called an "induction dose" of vaccine (62). This is defined as an intradermal injection of autologous melanoma cells without BCG, which was intended as a baseline test of DTH reactivity. Surprisingly, retrospective analysis showed that patients who received the induction dose 3–8 d before the administration of cyclophosphamide developed significantly higher DTH responses and had significantly longer relapse-free survival than patients who had been given the induction dose on the same day as cyclophosphamide administration. Thus, it seems plausible that the immunological success or failure of DNP-vaccine is determined very early in the course of treatment. If the induction dose is administered optimally, the magnitude of subsequent doses has only a minor effect. If the induction dose is administered suboptimally, manipulating the subsequent doses will not reverse immunological unresponsiveness.

8.12. Studies in Ovarian and Renal Cell Carcinoma

There are no practical impediments to trials of the DNP-modified, autologous vaccine in other cancers. However, given melanoma's reputation as being particularly immunogenic among human malignancies, some would speculate that the applicability of this immunological trick would be limited.

We have conducted two phase I trials in patients with ovarian cancer. In the first trial, 9 evaluable patients (10 total) with newly diagnosed adenocarcinoma of the ovary underwent standard debulking surgery plus six cycles of chemotherapy (taxol + platinum) prior to receiving DNP-vaccine. In the second trial, 13 evaluable patients (20 total) with bulky, chemotherapy-refractory disease were treated. We chose dosage schedule D (see Table 2) because it appeared to induce optimal DTH responses in the melanoma studies. Positive DTH (5-mm induration) to autologous DNP-modified tumor cells was elicited in 19 of 22 patients (median = 14-mm induration). More importantly DTH to *unmodified* tumor cells was induced in 17 of 22 patients and (median = 6 mm). DTH to autologous DNP-modified lymphocytes and to autologous unmodified lymphocytes was observed in only 6 of 22 and 0 of 22 patients, respectively.

Unexpectedly, a clinical response was observed as well: one patient, who had previously had excision of a peritoneal tumor, exhibited complete regression of a residual peritoneal mass by computed tomography (CT) and a concomitant fall in serum CA-125 from 65 to 6. Both the CT and CA-125 responses were maintained for 6 mo.

Ten patients were treated in a phase I trial of DNP-modified vaccine in renal cell carcinoma. The results were similar: 7 of 10 patients developed positive DTH to both hapten-modified and unmodified autologous cells.

Our results indicate that the requisite T-cell-mediated immune responses can be generated in patients bearing these two adenocarcinomas. Obviously, the demonstration of clinical effectiveness requires additional studies.

9. CONCLUSIONS

It is clear that a principle established more than 50 yr ago—that hapten modification can result in immune responses that are otherwise not possible—applies to tumor antigens. Thus, our studies on the autologous, DNP-modified vaccine have an impressive pedigree. The clinical trials performed so far—in melanoma, ovarian carcinoma, and renal cell carcinoma—show that the hapten system works with human tumor cells and suggest that the elegant hypotheses developed and supported by the basic scientists can explain the immunological results. The ultimate test of whether the DNP-modified vaccine is clinically effective is, of course, a randomized prospective trial. In the meantime, there is a great deal of productive work to be done. By incorporating other haptens, particularly those whose immunochemistry is different from DNP, second-generation cellular vaccines can be produced that should be more immunogenic. In addition, the effect of immunizing with defined tumor antigens, especially whole proteins, that have been hapten modified deserves to be investigated.

Haptens, which have taught us so much about how lymphocytes interact with proteins, both foreign and self, may cast some needed light on the dark corner of tumor immunology.

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Chaperone Proteins/Heat Shock Proteins As Anticancer Vaccines

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1. A SHORT HISTORY

The chaperone proteins, a family whose members include heat shock proteins (HSPs) and stress proteins, are among the most abundant proteins found in cells. The fundamental roles played by these proteins are so essential to cell survival that they have evolutionary origins as old as *Archaeabacter*. Despite the long history of their existence, scientists

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have only recently learned of the pivotal activities possessed by chaperone proteins, leading up to their use as anticancer vaccines.

R.J. Ellis and colleagues were probably the first to publish the term “molecular chaperone” (1–3) to describe any entity, usually a protein (or even a segment within an individual protein) that could nonsterically influence the proper folding pathway of a protein or polypeptide (such as a nascent polypeptide exiting a ribosome) by generally preventing that emerging protein from inappropriate interactions with other proteins. This would presumably allow the chaperoned protein to assume a native conformation at its lowest energy state. Information was just emerging that a number of the proteins referred to as “heat shock proteins” could indeed act as molecular chaperones (2,4). The remainder of this chapter will focus on this class of chaperones, but the reader should be aware that other molecular chaperones exist that are critical to the assumption of native confirmations of proteins and protein complexes within the cell (5).

The concept of stress-induced protein expression was introduced in the now-legendary experiments of Ritossa (6) where the *Drosophila buskii* third instar larvae were exposed to temperatures of 30°C instead of their typical 25°C laboratory environment. Salivary gland chromosomal “puffing,” indicative of areas of transcriptional activity, was evident in specific regions of chromosome 2L, whereas other normal transcriptional activity along the polytene chromosome was dramatically reduced. Similar results were obtained if dissected salivary glands were exposed to 2-4-dinitrophenol or sodium salicylate rather than heat shock, thus perhaps unwittingly demonstrating common response links between disparate stresses. It was over a decade before the *Drosophila* gene products (proteins) induced by heat shock were identified (7), opening the door to molecular analyses in a genetically amenable organism. Work in insect tissue culture soon followed (8,9) and eventually, mammalian HSPs were purified as well (10). Though it was not entirely clear what roles these proteins played in the cell, experiments had shown that the expression of HSPs correlated with cellular adaptation to stress, in this case, heat stress. Later, it became evident that a wide variety of stresses, such as anoxia, amino acid starvation, glucose starvation, oxidative stress, radiation, and even cold stress could lead to the expression of at least some of the HSPs, which were now thought of more broadly as “stress proteins” (reviewed in 11 and 12).

Eventually, the molecular functions of the stress proteins (stabilizing unfolded proteins, preventing/disassembling protein aggregates, refolding or condemning denatured proteins upon release from stress) were recognized as entwined with those functions expected of chaperones. In the case of chaperones, however, the duties were to assist in folding of nascent proteins and prevent their aggregation/denaturation in the absence of additional stress. Thus, the cytoprotective properties of stress proteins appeared to be related to their chaperone capacities (2,4,13–15).

2. CURRENT STATE OF THOUGHT

Even normal cellular conditions are rife with the possibility of protein aggregation, given the enormous concentration of intracellular proteins and other macromolecules (as high as 150–300 mg/mL, depending on cell type; reviewed in refs. 16–18). A nascent protein chain exiting a ribosome needs to rapidly fold in order to avoid coming in contact with other molecules before nonspecific interactions (usually of a hydrophobic nature) can take place, potentially rendering the nascent protein useless. This is where the heat

shock/stress proteins come into play, transiently binding to regions of exposed nascent peptide chain, allowing the thermodynamic properties of the new protein to drive its structural shaping, and preventing inappropriate interactions with other proteins until such time as those interactions become necessary (e.g., in the formation of multi-subunit complexes or stabilization of enzymes/receptors, etc., that await the arrival of ligand). Since these processes continually occur in the normal cell under nonstressed conditions, members of the heat shock/stress protein families are constitutively expressed at high levels under normal conditions, and their activities are analogous to those of chaperones, leading to the most generalized name for the heat shock/stress protein family. The literature on the topic of chaperones has grown vast. Numerous authors have reviewed the concepts of chaperoning polypeptides, protein folding, and the cell biological implications of chaperone activity and the stress response in much more extensive and sublime detail than will be provided here. For reviews from merely the past 2 yr the reader is referred to those for further information (19–29).

Technically, only proteins derived from genes that have heat shock elements (HSEs), which are targets for regulation by heat shock factor (HSF) transcription complexes, are true HSPs. Other genes may be transcribed and proteins translated as secondary consequences of heat shock (e.g., the “unfolded protein response”), but in the purest sense, those are not true HSPs. Nonetheless, the usage has become so commonplace that to make such fine distinctions may only serve to confuse; therefore, stress proteins that are not bona fide HSPs will be classified as such in this chapter. In general, however, we will attempt to refer to the proteins as “chaperones” as often as possible. Families of heat shock/chaperone proteins are often categorized by their relative molecular weights as determined by gel electrophoresis. Such familial organization and functional descriptions are shown in Table 1.

Other important mammalian chaperones that do not fit neatly into the categories of Table 1 include those of the endoplasmic reticulum such as calreticulin, calnexin, tapasin, and protein disulfide isomerase. These proteins, along with GRP94 and GRP78, are important in the folding and maturation of immunologically relevant molecules such as immunoglobulins and major histocompatibility complex (MHC) class I molecules. In more general terms, these proteins are critical members of the quality control apparatus of glycoprotein folding in the endoplasmic reticulum (ER) (21,30,31). Other chaperone/cochaperone proteins include the peptide-prolyl isomerases, the immunophilins, the cyclophilins, auxilin, etc. Also, an increasing number of cochaperones are being identified that appear to modulate the activity or outcome of chaperone-based protein folding. These include Hop (Heat shock protein70/90 Organizing Protein), Hip (Heat shock protein Interacting Protein), and members of the BAG-1 family. These cochaperones seem to compete for binding sites on the HSPs such as HSP70 and HSP90, leading to formation of hetero-HSP complexes, stabilization of HSP binding to ligands, or movement of HSP-bound misfolded proteins toward the proteosome for degradation (32).

3. CHAPERONE FUNCTIONS

How do chaperone proteins perform their duties? Current thought is that the intrinsic “fold-ability” of cellular proteins is built into them at the genetic/amino acid sequence level (33). Thermodynamically, the proteins seek the lowest energy state via assumption of an appropriate tertiary structure, generally by relegating hydrophobic amino acids to

Table 1
Families of Heat Shock/Chaperone Proteins

Family	Function	Unique Features	Cancer Vaccine	References
HSP110 GRP170	Aggregation deterrent Assist in protein folding	Relatives of HSP70 Can stably bind large proteins in long-lived manner	Yes	109–116
HSP100 Clp	Proteaseas; prevent and disassemble aggregates; assist HSP70 in refolding proteins	HSP104 necessary for prion activity in yeast	No	117–119
HSP90 GRP94/gp96	Folding and unfolding; stabilization of client “signaling” proteins; ER quality control; loading of peptides onto MHC I molecules	HSP90 binds HSF-1 to control its own transcription; earliest identified HSPs as anticancer vaccines; a buffer against genetic/mutational variation?	Yes	30,31,39, 120–123
HSC73, HSP72, mtHSP70/GRP78, GRP78, HSP71t	Folding, unfolding; intergranellar transport; clathrin uncoating; ER quality control	First HSP to be shown to bind peptides; utilizes ATP hydrolysis/ADP exchange for reaction cycle	Yes	20,48,49, 109,124, 125
HSP60 (chaperonin) GroEL TRiC/TCP	Folding, unfolding, refolding, sometimes specialized for particular proteins	Forms barrel-like structures with lids (GroES) that utilize ATPs to unfold/refold proteins; expression on tumor cell surfaces may have immunologic or metastatic consequences	No	20,97, 126–130
HSP40 DNA-J family	Assist HSP70 activities; involved in HSP70 ATP hydrolysis and nucleotide exchange	May have chaperone function; may require HSP70 cooperation	No	20,124, 125
sHSP (small HSP)	Stabilization and disassembly of aggregated proteins	Act as multimers; α -crystallin prevents aggregation of proteins in eye lens optical path; cancer status marker	No	131–133
HSP10 GroES Cpn10 EPF	Cap-like structure for HSP60 chaperonin; assist in folding; usually mitochondrial in eukaryotes	Early Pregnancy Factor has growth factor and immunosuppressive properties in serum of pregnant animals	No	134–138

the interior of the folded protein while allowing hydrophilic amino acids access to aqueous solvent. The challenges to reaching this tertiary structure begin as the nascent polypeptide chain exits the ribosome, and continue throughout the life-span of the protein. As incomplete protein chains depart the ribosomal complex, the polypeptide chains present stretches of exposed hydrophobic amino acids, which, if left unfolded or unprotected, are available for inappropriate contact with other proteins or even within the proteins in question. This inappropriate contact may lead to inactivation or aggregation of a protein even before it is fully synthesized. In addition, sampling of alternate folding pathways may lead to kinetic “traps,” rendering the proteins useless, or even deleterious if they are capable of nonspecifically aggregating other proteins. Segments of hydrophobicity also appear to be recognized by chaperone proteins. The chaperone proteins bind to the unfolded proteins, and protect them from unsuitable interactions; by cycles of binding and release, the chaperones give the prospective properly shaped proteins a sequestered microenvironment in which they get a chance to achieve thermodynamic stability. The protein structures continue to face challenges owing to cellular stress or mutation, which may lead to instability of a given protein’s three-dimensional structure. This could generate excessive fluctuations in the protein’s shape, exposing stretches or patches of hydrophobic amino acids that are likely to lead to aggregation. Again, the chaperone proteins recognize the exposed hydrophobic regions and bind to them for protection. It may be necessary to further unfold the misfolded proteins before attempting refolding, and in some cases, lead them down degradative pathways if the aberrant proteins are deemed unsalvageable. The decision-making process of degradation versus refolding malfolded proteins remains an enigma. Recent evidence suggests that the role of cochaperones of various types, such as Hip, Hop, and BAG-1, may be to compete for the direction of those outcomes by allowing or preventing the continued folding of a non-native protein. Ultimately, the chaperones are believed to lower the activation energy barriers to allow proteins to assume the proper folded shape while avoiding kinetic and thermodynamic traps. How chaperones accomplish this by what appears mechanistically to be no more complicated than binding and releasing hydrophobic segments of protein chain is still a mystery.

Perhaps the unifying molecular theme of chaperone proteins is their collective ability to recognize hydrophobic stretches of amino acids within a protein chain, either as a “clamp-on-a-string” such as HSP70, or as more of an ensemble, in the case of multiple members of the chaperonin complexes. This theme presumably extends down to the peptide level, as well, such that chaperones likely play an important role in intracellular and interorganellar peptide trafficking (such as in the generation of peptide-loaded MHC molecules). Such activities will be critical in defining chaperone-mediated vaccine effects, as described later.

4. CHAPERONE PROTEINS AS ANTICANCER VACCINES: EARLY DAYS

Thus, chaperone proteins are critical to cellular function, and are involved in the births, lives, and deaths of many cytosolic, organellar, and extracellular proteins. In that context, it is not surprising that chaperone proteins are important to the activities of the immune system, particularly in the assembly of immunoglobulins and MHC molecules, but the discovery that chaperone proteins could serve as vaccines was not an obvious corollary. In fact, that path is highly convoluted, and still under construction. It began largely with

the combined works of Lloyd Old, Albert DeLeo, Lloyd Law, Garrett Dubois, Ettore Appella, Stephen Ullrich, and Pramod Srivastava with their efforts to biochemically purify tumor antigens from primary rodent tumors. These groups used well-established chemically induced sarcomas as their models with the intent of identifying and characterizing tumor-specific transplantation antigens (TSTAs), tumor-associated transplantation antigens (TATAs), and tumor rejection antigens (TRAs). It was known at the time from transplantation studies that these tumors were immunogenic in syngeneic mice, but the immunity was specific to the original tumor, or at least was very limited to only a few tumor types (34). Serologic characterization implied that cell-surface molecules mediated the transplant immunity; i.e., antisera generated against tumor cells could lyse tumor cells in microcytotoxicity assays (35), but biochemical characterization was lacking. These investigators wanted to purify the agents of transplant immunity by brute-force biochemical criteria and identify the proteins that were the apparent antigens. In doing so they discovered that fractionated cytosolic preparations could adsorb the immune (cytotoxic) activity of the sera and could also provide transplantation immunity against the tumors of origin when injected as vaccines (36–38). Thus, the focus became biochemical, and molecular analyses of tumor antigens had begun.

Protein purification involves separation of the various substances that make up a cell away from each other until one finally obtains the desired component. Generally, this requires some sort of assay to determine the success of each step in the purification, and such assays are frequently enzymatic, immunological (i.e., Western blotting), or even visual (color, or spectral readings). In the case of the search for tumor antigens, the assays involved vaccinating mice with various fractions from the precipitation and chromatographic steps of the purification, followed by challenge of the mice with the original tumor. The mice that mounted immunologic responses against the tumor challenge (reduced tumor growth) were, presumably, vaccinated with active fractions. Those fractions were purified further and assayed similarly.

5. CHAPERONE PROTEINS AS ANTICANCER VACCINES: IDENTIFICATION OF HSP PEPTIDE COMPLEXES

What these researchers had collectively discovered were different members of the HSP90 family that were capable of protecting mice when tumor challenges were issued following preimmunization. The purified antigens would protect mice against tumor challenge from the tumor of the antigen's origin, but would not protect against challenge from another tumor, and vice versa (39,40). This implied a tumor-specific antigen and argued that such antigens were not shared but were unique among different tumor types. The perplexing part was that the particular antigens from different tumors appeared to be the same protein, in biochemical characteristics, in cross-reactivity with antisera raised against the antigens from each tumor separately, and ultimately, in amino acid sequence. This suggested that antigens from different tumors were indeed shared, but in this case, did not provide for a cross-reacting immune response. Upon amino acid sequencing of the “same-but-different” tumor antigens, they found that the proteins were apparently identical to newly identified members of the HSP90 family, including a previously identified chaperone protein known as glucose-regulated protein 94 (GRP94), which had been independently isolated and named by several other groups (e.g., endoplasmic Erp99, HSP108, etc.) (41–45). GRP94/gp96 was the endoplasmic reticulum paralog of

the cytoplasmic HSP90, abundant and ubiquitously expressed across phyla. Sequencing of the cDNAs for HSP90 and for GRP94/gp96 from both normal murine and tumor tissue indicated that each of the respective sequences were identical and nonpolymorphic (46). The researchers were faced with ubiquitously expressed, highly conserved, nonmutated antigens that could be purified from different tumor tissues, but could only provide immune benefit as vaccines against the tumors of their origins. These were not the ideal candidates for tumor-specific antigens. Despite the conundrum, Srivastava and colleagues continued studying GRP94/gp96 as well as other chaperone proteins and found that both HSP70 and HSP90 from tumors could generate protective immunity specifically against the tumors from which the HSPs were purified (47). These researchers speculated that there might be some low-molecular-weight (albeit very tightly bound) component that could engender the immune response (reviewed by Srivastava in 29). However, HSP vaccines were still a phenomenon without a mechanism. In time, it was shown that HSPs of the HSP70 family were capable of binding short (5–9 amino acid) peptides (48,49). This provided an explanation for the tumor-specific immunogenicity of the common protein antigen: tumor-specific, antigenic peptides could be associated or complexed with the chaperone proteins. The peptides, and not the chaperone proteins themselves, would be the source of the antigenicity (50).

6. CHAPERONED PEPTIDES AS ANTITUMOR ANTIGENS: THE EVIDENCE

Evidence that peptide antigens are associated with the chaperone proteins has been both direct and indirect, whereby researchers purified chaperones from cells containing known antigens, either well-studied naturally occurring antigens (51), including those from human tumors (52), viral epitopes (53–57), minor histocompatibility antigens (58), or artificial ones transfected or transduced into the cells such as β -galactosidase (58) or chicken ovalbumin (59,60). The presence of these known antigens was in some cases demonstrated by harvesting lymphocytes from vaccinated animals and testing them for cytotoxicity against target cells presenting the antigen of interest. In other cases, a more physical association of antigenic peptide with chaperone protein was demonstrated, whereby peptides were stripped from chaperone proteins and used to pulse target cells for lysis by antigen-specific cytotoxic T lymphocytes (CTLs). In Ishii et al. (51), the peptides were separated and identified as (precursor) epitopes for CTLs using specific cell lysis as an activity assay. In Meng et al. (57), the peptide of interest (a hepatitis B viral peptide) was directly isolated from patient liver samples and microsequenced to identify its origin.

Eventually, the physical association of peptide with chaperone protein was also demonstrated by crystal structure (61) following nuclear magnetic resonance (NMR) structural data on the HSC70 peptide-binding pocket (62). Also, peptides have been stripped or released from HSP preparations, which abrogated the vaccine efficacy (63), and specific peptides have also been used to replace the endogenous ones that were released, thus resulting in specific immunity following vaccination with the reconstituted chaperone-peptide complex (64–66).

Though specific antigenic peptides could be demonstrated to be complexed with the chaperone proteins, it was the lack of specificity, or promiscuity, of the peptide repertoire bound to chaperone proteins that was touted as a significant benefit when utilizing chaperone proteins as vaccines, particularly anticancer vaccines. Rather than focusing on one

or even a few specific peptides as antigens against a tumor, the chaperone proteins were presumed to potentially have contact with any peptide within the cell, probably resulting from proteosomal degradation. The relatively high mutation rates of tumors, coupled with their lack of corrective activities, should in theory lead to the generation of numerous mutated (and potentially antigenic) peptides that could be complexed with cytosolic chaperones. The “relay line” hypothesis was proposed, whereby peptides from degraded proteins would first be bound by HSP70 or HSP90 shortly after exit from the proteosome. The peptides might be “handed off” between the cytosolic chaperones, which would then deliver the peptides to the transporter associated with antigen processing (TAP) to gain entry into the ER. There, the peptides would be bound by GRP94/gp96 to be loaded onto MHC class I molecules prior to transit to the cell surface (67). Such passing of peptides down the line implies that there are increasing affinities for the peptides by each chaperone further down the line. Although this may be true in specific cases, it is not clear that situation exists for most or all peptides, since the contributions of cochaperones or other cofactors may influence peptide transit. Evidence for the relay line is scant (51), and does not take into account that in some studies of proteosome subcellular localization it appears that the proteosome actually interfaces with the ER, presumably at TAP, to perhaps directly pour peptides through into the ER (68). This has led to the “presentasome” concept, where the proteosome, cytosolic chaperone proteins, TAP, ER chaperones, and MHC molecules are all at least functionally linked with the common goal of loading peptides onto the MHC molecules (69). This situation may be analogous to the apparent nonlinear nature of chaperone-assisted protein folding, where it appears that nascent polypeptides are provided a protected folding environment by numerous (but not necessarily sequential) chaperone systems to accomplish the final goal, a native structure for the folded protein. In a similar fashion, peptides exiting the proteosome may need a similar protected environment to prevent further degradation, and the identity of the chaperone providing that protection may be less important than the presence of any chaperone capable of binding peptide. Since chaperones play a role in leading proteins destined for degradation to the proteosome, it may be the same chaperones gathering peptides as they exit the proteosome that brought the intact protein there in the first place. In this scenario, one can imagine peptides being passed amongst the various chaperones depending upon the relative affinities of the chaperones for particular peptides, the distance to be traversed, the status of the chaperones themselves depending upon the cochaperones present, etc. Instead of a relay with chaperones racing to deliver the peptide to the next member of the team, the movement of peptide into the antigen presentation system may more resemble the Harlem Globetrotters’ “Magic Circle,” with peptides appearing and disappearing among the members of the nearby chaperone group until the peptide finally passes through TAP, whereby similar “mechanisms” may exist inside the ER prior to loading of MHC class I molecules. The closing of the circle of protein folding and protein degradation as mediated by chaperones is somehow satisfying, with both mechanisms driven by biophysics, proximity, and modulated cochaperone interactions. Again, mechanistic details are lacking, which serves to underscore how little is truly known about intracellular peptide trafficking.

7. CHAPERONED PEPTIDES AS ANTITUMOR ANTIGENS: MULTIPLICITY OF ANTIGENICITY AND CROSS-PRIMING

From the above discussion it should be apparent that when purifying chaperone proteins from tumors (or any other tissue, for that matter), one is “catching them in the act” of transporting peptides. At least some of the intracellular peptides remain bound by the purified chaperones, and those peptides that are antigenic can now be utilized in the generation of an immune response. Thus, chaperone protein purification may be envisioned as preserving a peptide “snapshot” of the cellular protein pool. This leads to the doctrine of multiple antigenicity, whereby presumably numerous peptide antigens are escorted by tumor-derived chaperone proteins. One need not even know the identity or quality of the antigens, as that is a choice the immune system will make. In a vaccine setting, such antigenic multiplicity would in theory prevent the escape of specific individual antigen-negative cells. Though such escape variants have been demonstrated to exist in some melanoma peptide vaccine trials (70,71), in other cases it appears that tumor survival selection was due to lack of immune effect in resistant lesions rather than decreased presentation of a particular antigen (72).

As mentioned above, chaperone proteins have been purified from cells containing known peptide antigens, and via direct or indirect assays, those antigens have been demonstrated to have been associated with the chaperones. This has recently been shown for primary human tumors (melanoma) as well (52). In that study, it was found that T-cell responses to melanoma antigen did not depend on the human leukocyte antigen (HLA) haplotype of the tumor from which the HSP70 originated. Cross-priming via chaperone protein has been observed before in animal models (54,58), and this would be an obviously important benefit to chaperone protein antitumor vaccinations. In circumstances where there is no knowledge of particular antigenic peptides, vaccination of animals with tumor-derived chaperones has shown potent antitumor effects in both prophylactic and therapeutic settings. The growing list of tumor types to which chaperone protein vaccine therapy is amenable is impressive, crossing strain barriers and histologic origins of the tumors (29). In our hands, at least, we have not yet found a murine tumor type that was completely intractable to chaperone protein immunization (73 and unpublished data).

8. MULTIPLE CHAPERONE VACCINES

If individual chaperone protein vaccines are effective against tumors, would one vaccine containing numerous chaperone protein family members be better? The perceived advantages would be the probability of increasing the overall number of peptides available for antigen presentation because of multiple chaperone proteins, as well as the odds of increasing peptide diversity if members of the different chaperone families tend to escort distinct sets of peptides. Although their utilization has been suggested (74), part of the reason that such multichaperone vaccines are not commonplace lies in the difficulty of purifying sufficient quantities of multiple chaperones from a single source. Though such schemes are available (75,76), they are time consuming and generate numerous protein fractions requiring analysis prior to vaccine preparation. Our group set out to find a method that might enhance the rapid purification of multiple chaperones while keeping the workload and turnaround time manageable and acceptable. We have utilized

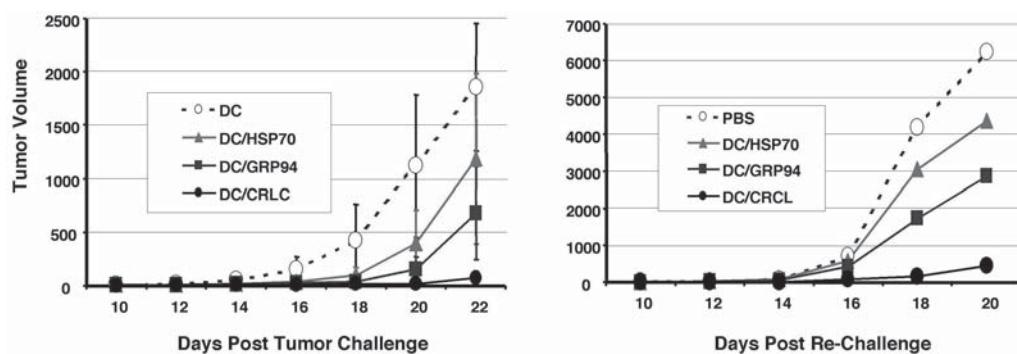


Fig. 1. Dendritic cells pulsed with chaperone proteins or with CRCLs (chaperone-rich cell lysates) generate potent antitumor immunity. Left panel: mice were inoculated with a lethal number of 12B1 leukemia cells on Day 0, and were vaccinated on Day 2 with 0.5 million syngeneic dendritic cells that had been left unpulsed or were pulsed with 12B1 tumor-derived HSP70, GRP94/gp96, or CRCL. Tumor volumes (in mm³) were measured thereafter. Right panel: tumor-free mice (3 of 12 from each DC/HSP70 and DC/GRP94 groups, and 9 of 12 from DC/CRCL group) were rechallenged with a lethal 12B1 tumor dose 80 d following the initial tumor inoculum. Age-matched naïve mice ($n = 4$, PBS) also received tumor challenge at that time. Tumor volumes over time were again measured in mm³.

a free solution-isoelectric focusing (FS-IEF) method to achieve the enrichment of the most commonly cited immunogenic chaperones (GRP94/gp96, HSP90, HSP70, and calreticulin) from tumor lysates in a limited number of fractions (73,77,78). We have termed these vaccines chaperone-rich cell lysates (CRCLs). CRCLs are clearly more effective as anticancer vaccines than unfractionated tumor lysate and are at least as effective as either purified HSP70 or GRP94/gp96 (73,77,78). The turnaround time on vaccine preparation can be as little as 24 h, and the yields are 1–2 mg of vaccine material per gram of murine or human tumor. Our group and others have also found that dendritic cells (DCs) pulsed with chaperone proteins are extremely effective cellular vaccines (30,60), capable of eradicating preexisting tumors in mice (73,78; Fig. 1). The interaction of chaperones with antigen-presenting cells (APCs) thus becomes the next topic of discussion.

9. CHAPERONE PROTEIN INTERACTIONS WITH PROFESSIONAL ANTIGEN-PRESENTING CELLS: RECEPTORS FOR HSPs

Our perspective so far has been from the inside of the cell, where chaperone proteins associate with intracellular peptides, to the outside of the cell, whereupon chaperone proteins obtained from the intracellular environment by extraction and purification may be utilized as multivalent, peptide-laden vaccines once removed from the cell. The next step is to return the vaccine to host cells, specifically, to those of the immune system involved in antigen presentation. Therefore, we will examine what is known mechanistically about the specific interactions of chaperone proteins with APCs that lead to immune effector cell responses.

Upon the discovery that chaperone proteins could bind peptides, the potential for chaperones to traffic in the currency of cellular immunity, i.e., antigenic peptides, was

also realized. The implication for peptide-carrying chaperones was that the peptide must somehow get inside an APC, probably via a receptor that could bind the chaperone protein. This was predicted by Srivastava et al. (67), and putative receptors (79) or coreceptors (80) have been identified on cell surfaces of APCs. Binder et al. (79) demonstrated that CD91, also known as the α 2-macroglobulin receptor and the low-density lipoprotein (LDL) receptor-related protein, appeared to be a receptor on macrophage for GRP94/gp96; subsequent study by Basu et al. (81) indicated that macrophage and DC CD91 was a common receptor for not only GRP94/gp96, but also HSP70, HSP90, and calreticulin. α 2-macroglobulin itself has even been shown to bind peptide and elicit CTL responses, at least in vitro (82), which may have significant ramifications for antigen presentation. In human monocytes, HSP70 was shown by Asea et al. (80) to act as a cytokine via a CD14-dependent pathway, implicating CD14 as a “co-receptor” for extracellular HSP70. Curiously, Kol et al. (83) also implicated monocyte CD14 as a receptor for HSP60, one of the chaperones not currently demonstrated to have antitumor-immunizing effects. However, Ohashi et al. (84) established APC Toll-like receptor 4 (TLR-4) as an APC receptor for HSP60, as well. Recently, Vabulas et al. (85) have shown that GRP94/gp96 utilizes the TLR pathway (TLR2/4) to activate DCs, but it is not known if this pathway involves antigen delivery as well. Also, the scavenger receptor, CD36, has also been shown to bind GRP94/gp96 (86). To further confuse the issue, Berwin et al. (87) provided recent evidence in macrophage cells that GRP94/gp96 can mediate specific immunity independent of CD91. Thus, the story of specific receptors on APC surfaces for chaperone proteins is not at all clear, and will continue to be an active and contentious area of research.

10. CHAPERONE PROTEIN INTERACTIONS WITH PROFESSIONAL ANTIGEN-PRESENTING CELLS: PHENOTYPIC EFFECTS ON APCS

Although the specific receptor(s) for chaperone proteins await clarification, the phenotypic effects of exogenous chaperones on APCs are legitimately recognizable, if not entirely uniform. Professional APCs such as macrophage cells and DCs upregulate expression of MHC class I and II molecules, costimulatory molecules CD80/86 (B7-1 and B7-2), and CD40 (78,88,89) when these APCs are exposed to exogenous chaperone proteins, whether those chaperones are tumor derived or have been isolated from normal tissues, i.e., devoid of tumor-peptide antigen (90). Such stimulated APCs will also secrete a plethora of pro-inflammatory cytokines, including TNF- α , IL-1 β , IL-6, and IL-12, presumably triggered via NF- κ B-promoted pathways (78,89). It appears that exogenous chaperone proteins are at the crux of both adaptive and innate immune responses in that they are capable of supplying peptide antigens in an extraordinarily effective manner whereas their mere presence primes the immune system for action thru the activity of APCs. Exogenous chaperone administration results in APC activity reminiscent of bacterial lipopolysaccharide (LPS)-induced APC activation, and the pathways and signal transduction cascades may be similar. The chaperone proteins show high sequence homology throughout species, and bacterial and viral HSPs are potent antigens in mammalian hosts. The release of “self” chaperones during cell lysis, in a situation where infected cells or tumor tissue was undergoing necrosis, may prompt a hard-wired response from the immune system that has been evolutionarily “trained” to react to the extracellular presence of chaperones. Speculations on the relationships between bacterial LPS, HSPs,

CD91, and the innate/adaptive immune response have been elegantly reviewed recently (29).

11. CHAPERONE PROTEIN INTERACTIONS WITH PROFESSIONAL ANTIGEN-PRESENTING CELLS: NATURAL ADJUVANTS AND “DANGER SIGNALS”

The aforementioned “hard-wired” immune response to chaperone proteins makes them excellent candidates for providing “danger signals” to the immune system (91,92). Much has been made of this in defining the immunological relevance of apoptotic vs necrotic cell death. Necrotic cell death has been implicated as leading to immunological consequences, whereas apoptotic cell death is often regarded as silent or “bland” to the immune system, although this is somewhat controversial. Expression or release of chaperones during cell death leads to activation of APCs (93–96), as is apparently the case during necrosis. However, our work has demonstrated that at least some stressed apoptotic tumor cells may also express HSPs on their cell surfaces, which is coincident with emergent immunogenicity of those cells that were previously nonimmunogenic (97). Moreover, we have shown that addition of exogenous chaperone proteins (derived from normal tissue) to cells undergoing chemotherapy-induced apoptosis leads to DC activation and generation of a potent immune response against the tumor (90,98). Thus, the presence or absence of chaperone proteins in the extracellular milieu or on cell surfaces may be critical in determining the immunological outcome of cell death. This is consistent with the view that chaperone proteins represent the first adjuvants of mammalian origin (64,69) and makes the use of chaperone protein “therapy,” an interesting prospect from the perspective of a vaccine that delivers antigenic material as well as from the perspective of an adjuvant that incites activation of APCs regardless of the source of the antigenic material (e.g., tumor cells undergoing chemotherapy-induced apoptosis).

12. CHAPERONE PROTEIN INTERACTIONS WITH NON-APCS: REGULATORY EFFECTS

As might be expected of immunological phenomena, there are likely to be checks and balances concerning chaperone-induced APC activation. One curious mechanism for this may be the roles played by platelets, which appear to bind GRP94/gp96 via CD91 and CD36. Binding of the chaperone to platelets results in a downregulation of DC activation/maturation, which may protect the body against the development of chronic inflammation (99). It has been demonstrated previously (100) that “excess” GRP94/gp96 (i.e., vaccination with more than some limiting quantity of the chaperone) resulted in a loss of immunization effect. Whether or not binding of the chaperone by non-APCs can regulate the overall systemic immune response remains to be seen, but it is tempting to speculate that perhaps excess chaperone may enter the bloodstream (after saturation of dermal APC chaperone receptors). In turn, this may result in chaperone binding to platelets, leading to some control over the extent of APC-driven immune consequences.

13. CLINICAL TRIALS OF CHAPERONE PROTEIN VACCINES

In the first published study, Janetzki et al. (101) reported on 16 patients with various advanced malignancies, vaccinated with GRP94/gp96 that was prepared by the Srivastava

group's purification procedure. Significant toxicities or autoimmune reactions were not observed. Immune responses were evaluated using ELISPOT assays of peripheral blood T cells. Immunization with autologous GRP94/gp96 elicited MHC class I-restricted, tumor-specific CD8+ T cells in 6 of the 12 immunized patients.

Antigenics Inc. (New York, NY) has developed a clinical GRP94/gp96 vaccine termed HSPPC-96, or Oncophage (102). Data generated from clinical trials evaluating HSPPC-96/Oncophage against a variety of cancers have been reported at recent American Society of Clinical Oncology (ASCO) and American Association of Cancer Research (AACR) conferences. The major limiting factor for producing Oncophage from a patient's tumor is the ability to purify enough GRP94/gp96 from the autologous tissue. Oncophage is manufactured in a 10-h process from autologous tumors. The minimum tissue required to produce enough vaccine for a course of treatment is between 1 and 3 g, and patients have been immunized either subcutaneously or intradermally weekly for 4 wk and then every two to four weeks for another three to five vaccinations. In the reported trials, HSPPC-96 vaccine consisted of 2.5, 5, 10, 25, 50, or 100 µg of purified GRP94/gp96. Generally, adequate protein was purified to complete the vaccination schedules, but the yield of vaccinating material was dependent on the type of tumor used. Adequate Oncophage (percent successful attempts) was manufactured from the following tumors: colorectal carcinoma, 100%; renal cell carcinoma, 98%; melanoma, 90%; gastric cancer, 71%; pancreatic cancer, 30% (102). The lower success rates with gastric and pancreatic cancers are not surprising in that the digestive enzymatic capacities of these cells make any purification difficult at best.

Oncophage is in phase I/II trials in Italy (Istituto dei Tumouri, Milan) for colorectal cancer and melanoma. Parmiani et al. (103) reported that 2 of 34 patients with metastatic melanoma immunized with autologous GRP94/gp96 achieved durable CRs (459+ and 582+d), and in 3 patients the disease stabilized following vaccination (for 405, 562+, and 382+d). ELISPOT assays were performed with material from 12 HLA-A2 patients (including 3 out of 5 responding patients). Peripheral blood lymphocytes (PBLs) from immunized patients were stimulated with autologous melanoma cells, HLA-A2 allogeneic lines, or gp100/MART-1 peptides. The PBLs of two of three clinically responding patients showed an increase in number of spots when tested with autologous tumor, and PBLs of two patients showed responses when stimulated with allogeneic melanomas. Three of the responders (but none of the six nonresponders) had increased numbers of spots in response to MART-1_{27–35} peptides following immunization. Mazzaferro et al. (104) presented data on patients immunized with autologous GRP94/gp96 after resection with a curative intent of colorectal liver metastases. A positive DTH response was observed in 4 out of 29 patients. Nine HLA-A*0201 patients showed a transient but significant increase in T-cell recognition of allogeneic HLA-matched colon cancer lines and/or CEA, EPACM, and Her-2/Neu peptides.

Oncophage is in a pivotal phase III clinical trial for renal cancer at multiple sites worldwide with the goal of enrolling at least 500 patients who are randomized to have surgical removal of the primary tumor followed by Oncophage or surgery alone. This study was initiated on the basis of encouraging results from pilot phase I/II studies in patients with renal cancer. Amato et al. (105) immunized patients with Oncophage prepared from renal cell carcinoma starting 4 wk after nephrectomy. Responding patients and those with stable disease continued on Oncophage immunization, whereas in those that progressed, subcutaneous IL-2 was added. Eight of 25 evaluable patients remained

on vaccine alone (1 CR, 1 partial response [PR], 6 stable disease [SD]) whereas in 9 patients, IL-2 was added (8 patients were off study). In October 2001, the Food and Drug Administration designated Oncophage as a fast-track product in the United States for renal cell carcinoma.

A phase I/II trial has been carried out in Germany for gastric cancer (Johannes Gutenberg-University Hospital, Mainz). Hertkorn et al. (106) reported that immunization of gastric cancer patients with autologous tumor-derived GRP94/gp96 resulted in an expansion of CD8+CD45RO+ memory T cells in 8 of 11 patients, and an increase in CD8+ T cells with specific TCR-V-beta subtypes was seen in 2 of 9 evaluated patients.

Finally, in a study from Memorial Sloan Kettering in New York, patients were vaccinated with autologous tumor-derived GRP94/gp96 following surgical resection of pancreatic adenocarcinomas (107). From 15 patients that underwent pancreaticoduodenectomy, in only 3 cases was there enough protein purified to carry out vaccinations. This low yield, as mentioned earlier, was attributed to the large amounts of proteases in pancreatic tissue. One of the three immunized patients showed an increase in CD8+ T-cell frequency against autologous pancreatic adenocarcinoma as assessed by ELISPOT.

HSP70, prepared by immobilized-ADP chromatography has also been used to immunize patients. Reed Sporn et al. (108) obtained tumor cells from 11 chronic lymphocytic leukemia patients by leukapheresis, and from 2 low-grade B-cell lymphoma patients by lymph node biopsies. Patients received intradermal injections of 10, 25, 50, or 100 µg weekly for 4 wk and then monthly for 3 mo. DTH responses were not observed in any of the 13 immunized patients, whereas a transient increase in HLA-restricted tumor-reactive CD8+ cells (as assessed by interferon- γ ELISPOT assays) was seen in 4 of the 8 patients upon whom analysis was completed.

As all of these clinical trials are in their infancy, it is premature to draw conclusions on the efficacy of HSP vaccines in humans; it is apparent, however, that GRP94/gp96 and HSP70 vaccines are safe and well tolerated with minimal toxicity to the patient. Furthermore, in what might be considered one of the more contentious issues surrounding the use of these vaccines in patients, no autoimmune reactions have been detected in immunized patients.

14. CLOSING REMARKS

There is great excitement and anticipation in the field of cancer vaccines that chaperone protein-based immunotherapy may provide an important new element in cancer treatment. From an immunological point of view, the vaccines have qualities that make them excellent choices: the chaperone proteins are relatively abundant, they escort a diverse repertoire of antigenic peptides as part of their chaperone duties, and their very presence stimulates APCs to mature and activate. In clinical trials, such vaccines have generated positive responses in patients who are immune compromised from prior therapy or tumor burden. In addition, these clinical trials have demonstrated the safety and tolerability of chaperone vaccines, indicating that the vaccines could be used in earlier treatment settings without compromising patient safety. Finally, the prospects of combining chaperone protein vaccine therapies with other conventional or experimental therapies need to be explored. These would include surgical resection (as an obvious supply of the vaccine start material), chemotherapies that have minimal impact on the immune system, and immune-boosting adjuvant therapies. Clearly, tumor-derived chaperone proteins are prime candidates as antigen sources for loading of and activating DCs

to generate effective cellular vaccines. There should be hope that anticancer vaccines of high specificity, high activity, and high tolerability will be available as personalized therapy in the near future.

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Dendritic Cell Vaccines

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1. INTRODUCTION

Development of vaccines that led to the eradication of major infectious diseases (e.g., smallpox) is one of the greatest success stories in modern medicine. Molecular identification of tumor antigens has ushered in a new era with optimism for similar success against cancer (1–3). However identification of tumor antigens alone is not sufficient for producing effective vaccines. This is perhaps best exemplified by chronic viral infections such as the human immunodeficiency virus (HIV), for which the antigenic targets have been known for some time. Vaccines are composed of both antigen(s) and adjuvant(s). Effective vaccines also require attention to vaccine delivery and vaccine adjuvants, or enhancers of immunity. These adjuvants play a critical role in determining both the quantity and quality of the immune response to the antigen (4). Dendritic cells (DCs), nature's adjuvant, are antigen-presenting cells (APCs) specialized to initiate and regulate immunity (5,6). Here, I will discuss the scientific basis for targeting DCs to harness the immune system against cancer. Use of these cells in cancer immunotherapy has been bolstered by improved understanding of DC biology and presentation of tumor antigens *in vivo* (6–9).

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2. RATIONALE FOR TARGETING DENDRITIC CELLS IN CANCER IMMUNOTHERAPY

In contrast to vaccines for the prevention of infections, cancer vaccines are administered in a therapeutic setting, to eradicate antigen-bearing tumor cells already present in the host (1,10). The fundamental premise behind clinical approaches for DC-mediated immunization in cancer is that the limiting defect in natural antitumor immunity is at the level of antigen presentation. A growing body of literature points to defects in DC activation or maturation in DCs from the tumor bed of patients (11). As DC maturation is a critical step for the initiation of effector T-cell responses, defects in DC maturation, at a minimum, may impair activation of antitumor immunity *in vivo*, and may help promote the development of tolerance mechanisms, including regulatory T cells (12). Targeting tumor antigens to DCs, either *ex vivo* or *in vivo*, therefore allows an opportunity to bypass these defects in antigen presentation, and takes advantage of the many specialized features of DCs as potent APCs (5,13).

3. DCS AS NATURE'S ADJUVANT

DCs are potent stimulators of T-cell responses and T-dependent antibody formation in tissue culture (14,15). Relatively few DCs and relatively low doses of antigen are required to elicit high levels of lymphocyte proliferation and differentiation. Initially, because DCs had to be isolated directly from lymphoid tissues (or, in the case of humans, from blood), the scarcity of these cells imposed a serious limitation on DC research. A major advance has been the development of new tools to obtain DCs in large amounts by culture of either CD34+ proliferating progenitors or CD14+ monocytic precursors (16–18). These DCs are charged or “pulsed” with antigens, which they efficiently process and display as major histocompatibility complex (MHC)–peptide complexes. Antigen-pulsed DCs can be placed into culture with lymphocytes, whereupon T cells begin to proliferate and to produce lymphokines and various cytotoxic molecules. Primary antigen-specific responses can be difficult to achieve in short-term (1 wk) culture, because the initial number of antigen-responsive cells is so low (<1 in 10⁵ lymphocytes), but mature DCs rapidly induce recall responses to many antigens. The potency of DCs in stimulating T cells *in vitro* reflects both their specialized ability to capture and present antigens and the effects of other costimulatory molecules and cytokines that enhance T-cell stimulation.

3.1. In Vivo Evidence for DCs As Strong Adjuvants

DCs can be exposed to an antigen either *in vivo*, by introducing the antigen directly, or *ex vivo*, by pulsing the cells with antigen while they are in culture (19). After antigenic proteins are given to mice, DCs are found to be the main cells capturing antigen in an immunogenic form (20). When mice are challenged with microbes, DCs also are the principal cells producing the key protective cytokine interleukin-12 (IL-12). *Ex vivo*-activated DCs can prime recipient animals in an antigen-specific manner, allowing them to respond to an antigenic challenge within a week. These DCs migrate to the recipients' lymph nodes and lodge in the T-cell areas, sites through which lymphocytes enter the lymph nodes via high endothelial venules. This movement positions the DCs in a seemingly ideal niche to select antigen-specific T cells when the latter percolate through the node (21). Such selection can be observed directly *in situ*: following activation in contact

with DCs, the T cells leave the lymph node, freeing them to fight infections and tumors. Some also become memory T cells, a response whose mechanism remains to be unraveled.

Inaba and colleagues demonstrated that the injection of DCs, charged with antigen ex vivo, could sensitize normal mice to protein antigens (19). This seminal work also suggested that using DCs directly as a vaccine might best circumvent the problem of variable in vivo DC targeting. The immunogenicity of antigens delivered on DCs has now been demonstrated in several human studies. Indeed, single sc immunization of healthy volunteers with $2\text{--}4 \times 10^6$ antigen-loaded mature monocyte-derived DCs rapidly expanded CD8+ and CD4+ T-cell immunity (22). A single boost several months later led to expansion of cytotoxic T lymphocytes (CTLs) with increased affinity against viral peptide (23). Thus DCs can lead to both quantitative and qualitative enhancement of CD4 and CD8+ T-cell immunity in humans.

There is a large body of literature involving animal models of tumor immunity in which DCs loaded with tumor-associated antigens (TAAs) are able to induce protective antitumor responses. When tested, DCs can be superior to other vaccination strategies (24). There also are reports in which DC immunization produces significant therapeutic immunity to established tumors (reviewed in ref. 25). A number of trials have now utilized TAA-loaded DCs as vaccines in humans. Initial pioneering studies involved patients with lymphoma and melanoma (26,27). Some clinical and immune responses (T-cell proliferation and delayed-type hypersensitivity [DTH]) without any major toxicity have been observed. More recent DC vaccination studies put further emphasis on the elicited immune responses and have included control antigens for CD4+ and CD8+ T-cell responses (28–31). Several of these studies are reviewed elsewhere in this handbook. Inclusion of control antigens helps to verify that the DCs are immunogenic, and that the patient's immune system is competent to mount an immune response. The Erlangen group demonstrated that T-cell immunity to both control antigens (viral peptide and bacterial protein) and melanoma peptide can be induced, even in patients with advanced stage IV melanoma, by vaccination with antigen-pulsed, mature monocyte-derived DCs (28). Furthermore, when these DCs were loaded with MHC class II-binding melanoma peptides, strong tumor-specific Th1 responses were elicited. Interferon (IFN)- γ -secreting Th1 cells are likely to be critical for more effective and long-lasting antitumor immunity (32). DCs derived from CD34+ progenitors and pulsed with control antigens and multiple melanoma peptides, induce primary and recall immune responses detectable directly in the blood in patients with stage IV melanoma (29). It was observed that the level of immune responses in the blood correlated with early outcome at the tumor sites, thus providing further stimulus for the idea that the measurement of immune responses in the blood helps evaluate vaccine efficacy.

For purposes of vaccine design, it may be most straightforward to target antigens selectively to DCs *in situ*. This approach has attracted particular attention, with the discovery that certain cytokines (e.g., FLT3-ligand and granulocyte-macrophage colony-stimulating factor [GM-CSF]) can mobilize DC progenitors or promote DC recruitment *in situ* (33). Targeting specific antigens to DCs *in vivo* has been achieved through the DEC-205 receptor (CD205), which mediates antigen uptake and processing in DCs. Crucially, induction of immunity also requires a stimulus that matures the DEC-205+ DCs (34). Antigen presentation sets the stage for antigen-specific T-cell recognition, but maturation controls the T-cell response. Therefore, vaccines must not only contain the

requisite antigens to initiate protective immunity but also provide stimuli to promote DC maturation.

4. ASPECTS OF DC BIOLOGY OF IMPORTANCE TO TUMOR VACCINES

4.1. Antigen Presentation on MHC Class I Products, Including the Exogenous Pathway

The presentation of vaccine antigens on MHC class I is needed to activate CD8+ CTLs, which are critical for protective antitumor immunity. The classical, “endogenous” pathway for presenting peptides on MHC class I products begins when endogenously synthesized proteins are clipped by the proteasome, and peptide fragments are transported via transporters associated with antigen presentation (TAPs) into the rough endoplasmic reticulum (13). There, the resulting peptides bind the peptide-binding grooves of newly formed MHC class I products, and the MHC–peptide complexes exit via the Golgi apparatus to reach the surface for presentation to antigen receptors on T cells. DCs are proving to be quite specialized in their capacities to form MHC class I–peptide complexes, which go beyond the classical endogenous pathway summarized above (35). One specialty is to about what is referred to “exogenous presentation” or “cross-presentation” (36). These pathways act, respectively, on proteins derived from immune complexes or inactivated microbes, or on antigens originally synthesized in other cells, which then “cross” to the MHC products of DCs. As tumors themselves are poor APCs, this pathway may be of special import for generating antitumor killer T-cell responses *in vivo*. Several DC receptors lead to MHC class I–peptide complex formation via the exogenous pathway. These include the Fc γ R, which binds immune complexes and antibody-coated tumor cells; the integrin $\alpha\text{v}\beta 5$ and the phosphatidylserine receptor, which bind dying cells; and various receptors for heat shock proteins (35,37–39). Subsequent delivery of antigen into the cytosol is postulated to require a transporter that allows macromolecules to escape the endocytic vacuole. Once in the cytoplasm, proteins may be subject to the newly recognized heightened capacity of maturing DCs to polyubiquitylate proteins (40). Ubiquitin conjugation marks the proteins for efficient proteasomal processing. The ability of DCs to cross-present antigens from dying cells has prompted several clinical studies loading DCs with tumor lysates or apoptotic bodies (41–44). More recently, the recognition that Fc receptor mediated targeting can boost cross-presentation of tumor antigens has encouraged the use of immune complexes and antibody-coated tumor cells as sources of antigen in clinical studies (38,45–47). It is anticipated that additional DC specializations will be found for increasing their efficiency in MHC class I–peptide complex formation via this pathway. It should be noted that, although many investigators use the terms “exogenous pathway” and “cross-presentation” to refer exclusively to presentation on MHC class I, DCs simultaneously present exogenous proteins and cellular antigens on MHC class I and II (48). Thus, in these pathways, CD4+ helper T cells can also be activated to amplify the CD8+ T-cell responses.

4.2. Antigen Presentation on MHC Class II Products

The MHC class II pathway, which forms MHC–peptide complexes to be recognized by CD4+ helper T cells, is particularly efficient in DCs. For example, when a protein is delivered to DCs via phagocytosis of cellular debris, the formation of MHC II–peptide complexes is actually several log more efficient than when preprocessed peptides are

loaded (48). DCs have many candidate receptors for dying cells, but active receptors in vivo remain to be identified. Conversely, several DC-restricted uptake receptors are known for which natural ligands remain to be identified. One example is the DEC-205 (CD205) uptake receptor, which traffics in a distinct way through DCs and greatly enhances antigen presentation relative to other adsorptive endocytic receptors (49). DEC-205 can recycle through the acidic late endosomal/lysosomal vacuoles in maturing DCs, compartments that are enriched for MHC class II molecules and proteinases like the cathepsins that mediate antigen processing and MHC class II-peptide complex formation (49).

These complexes, once formed, are transported to the DC surface within distinctive nonlysosomal transport vesicles (50). The vesicles contain both the MHC-peptide complexes, recognized by the T-cell receptor, and the CD86 molecules, required to costimulate T-cell growth. Upon arrival at the DC surface, the processed antigen and CD86 remain coclustered in aggregates that contain so-called tetraspannin membrane proteins (51). This situation seems ideal to set up immunologic synapses between DCs and the T cells that they activate. At this final mature stage, the DCs silence transcription of MHC class II products (whose genes are activated by the transcriptional activator CIITA) and shut down much of their endocytic activity, while actively presenting antigens captured in the periphery or vaccine site at lymphoid tissues (5). CD4+ T cells, particularly of the Th1 type, are thought to be important for antitumor immunity. The ability of DCs to elicit potent CD4+ T-cell responses and polarize responses to Th1 is therefore valuable to their use as adjuvants (32).

4.3. DC Maturation

In the absence of a perturbation such as infection or vaccination, most DCs remain at an immature stage of differentiation (52). To exploit DCs in vaccine design, the vaccine must not only provide protective antigens that are captured by DCs; it must also induce DC maturation. Immature DCs can capture antigens, but they must differentiate or mature to become strong inducers of immunity. DC maturation is the control point that determines whether an antigen is to become an immunogen (12). There are two well-studied classes of maturation stimuli. One class is provided when the microbe or vaccine signals DCs through toll-like receptors (TLRs) (53); a second class is provided by lymphocytes and other cells (either T, B, natural killer [NK], natural killer T [NKT], platelets, or mast cells) that deliver tumor necrosis factor (TNF)-type signals to the DCs.

Many defined microbial products initiate DC maturation through TLR signaling. DCs can produce particularly high levels of immune-enhancing cytokines like IL-12, IFN- α , and even, in some situations, IL-2. Over longer periods, DCs mature to become strong adjuvants for T-cell immunity. Expression of specific TLRs can be high in DCs, particularly TLR9, which responds to microbial DNA, and TLR3, a receptor for double-stranded RNA (53). TLRs can also respond to particular small molecules, like specific CpG deoxyoligonucleotide sequences, or to complex microbial macromolecules like DNA. TLRs signal through the MyD88 adaptor protein to trigger cytokine release from different cell types. However, DC maturation through certain TLRs is also influenced by a MyD88-independent mechanism that will be important to identify and manipulate. TNF family members that stimulate DCs include TNF itself, Fas ligand (FasL), CD40 ligand (CD40L), and TRANCE (RANKL). These molecules are expressed in a membrane-bound form by activated T cells and signal the corresponding activating subclass of TNF receptors (TNF-Rs). Possibly, different maturation stimuli (TLR vs TNF-R signaling) have different

consequences for DCs. Full expression of some DC functions, such as IL-12 production, may also require concerted signaling by both of these receptor types.

DC maturation is an intricate differentiation process whose different components may be under separate control. Antigen processing and presentation are regulated at several levels, notably through the control of intracellular proteinase activity. Thus, maturation diminishes the level of the cysteine protease inhibitor cystatin C within the endocytic system, permitting increased catabolism of the invariant chain by cathepsin S, and promoting the binding of antigenic peptides to MHC class II molecules (54). The expression of CD40 and other T-cell interaction molecules is also enhanced by maturation. Signaling through CD40, induced by CD40L on activated T cells, mast cells, and platelets, leads to the production of DC cytokines and chemokines and enhances DC migration and survival. Maturing DCs alter their expression of the costimulatory molecules CD80 and CD86 and of TNF family members, all of which can influence the extent and quality of the immune response. Maturing DCs also reshape their repertoire of chemokine receptors (55). Mature DCs lose CCR5 and CCR2, which respond to chemokines in an inflammatory site, but gain CCR7, which responds to chemokines in the lymphatic vessels and lymphoid organs.

4.4. DC Mobilization

DC numbers can be increased tenfold using cytokines like FLT-3L (56), whereas DC differentiation from nonproliferating precursors can be influenced by other hematopoietins (GM-CSF, IL-4) and interferons. The requisite chemokine receptors for DCs to traffic into a vaccination site may vary, with CCR5 and CCR2 responding to MIP-1 and monocyte chemoattractant proteins in interstitial compartments. For vaccines administered into the skin and muscles, migration to lymph nodes requires afferent lymphatics, but the DC–lymphatic interaction is still poorly understood (57). Migration of antigen-bearing DCs to T-cell areas of lymph nodes are influenced by cysteinyl leukotrienes and transporters of the multidrug resistance family, as well as the distinct TREM-2 signaling molecule (57,58). Once in the T-cell area, DCs are short-lived, apparently dying within a few days. Their life-span can be prolonged through membrane-bound TNFs on the T cell, e.g., CD40L and TRANCE (RANKL). Promoting DC migration to lymphoid tissues provides important opportunities to enhance efficacy of DC vaccines.

4.5. Antigen Presentation on CD1 Glycolipid-Binding Molecules

DCs express the known members of the CD1 family of antigen-presenting molecules, but individual CD1 molecules can be restricted to subsets of DCs. CD1a is typically found on epidermal Langerhans cells (LCs) in skin, whereas CD1b and c are expressed on dermal DCs. CD1 molecules present microbial glycolipids, but in addition, CD1d on DCs can efficiently present a synthetic glycolipid, α -galactosylceramide, to activate distinct lymphocytes with restricted T-cell repertoire (NKT cells) (59). Indeed, DCs are superior to monocytes for the activation of freshly isolated human NKT cells (60). Following recognition of glycosphingolipid on CD1d, NKT cells orchestrate the production of large amounts of cytokines from several cell types and have the capacity to act as adjuvants for T-cell-mediated immunity. Function of CD1 molecules and presentation of tumor-derived glycolipid ligands on DCs is an area of active investigation.

Table 1
Some Variables to Optimize Clinical Application of DC-Based Cancer Vaccines

DC Related:

- Subset of DCs
- Type of maturation signal
- Route of administration, frequency, dose, schedule, and duration
- Choice of antigen preparation and method of loading
- Optimizing migration of DCs
- Quality control of manufacturing process

Clinical Aspects:

- Combination with other therapies
 - Patient selection
 - Immune monitoring
 - Trial designs and end points
-

4.6. Activation of Natural Killer Cells

Several initial pieces of information are in place with respect to DCs and NK cells. DCs can be targets for NK cells and therefore should also have ligands for activating NK receptors (61,62). DCs express high levels of requisite costimulators for NK cells, e.g., CD48 that costimulates NK cells via 2B4. A first report shows that DCs can directly activate NK cells in mice, probably via contact-dependent mechanisms (62). DCs can release large amounts of the key cytokines, like IL-12 and IFN- α , which improve NK function. IL-2 induced from T cells by DCs could also expand NK numbers. Therefore even though NK cells are part of the innate immune response, they can be mobilized by DCs to enhance tumor cell killing and avoid key tumor escape mechanisms.

4.7. Generation of Antibody-Forming B Cells

Classically, DCs enhance antibody formation by promoting the formation of antigen-specific CD4+ helper T cells, which induce antigen-specific B cells to proliferate and make antibody. *In situ*, IFN- α enhances T-dependent antibody formation, isotype switching, and memory (63). To obtain this result, DCs are the only cells that need to express the requisite type I interferon receptors. DCs can have direct effects on B cells that greatly enhance immunoglobulin (Ig) secretion and isotype switching, including the production of the IgA subclass of antibodies, which contribute to mucosal immunity (64,65). Consequently, vaccines targeting DCs could theoretically promote both antitumor B-cell and T-cell immunity.

5. SOME PARAMETERS FOR OPTIMIZING DENDRITIC CELL VACCINES

As noted earlier, several groups have now demonstrated the feasibility of DC vaccination in cancer. These studies have shown that injection of these DCs is well tolerated, at least in the short term, and that antigen-specific CD4 and CD8+ T-cell responses can be elicited in these patients. Although the results with antigen-bearing DCs are encouraging, DC vaccination is at an early stage, and several parameters need to be established (Table 1).

5.1. The Subsets of DC

Prior studies have identified distinct DC subsets in humans from analyses of skin DCs, DCs generated in vitro by culture of CD34+ stem cells, and blood DC precursors (reviewed in 66). Human skin contains epidermal LCs, characterized by the expression of CD1a and by Birbeck granules, and interstitial (dermal) DCs, which lack Birbeck granules but express coagulation factor XIIIa. These two subsets also emerge in cultures of stem cells with GM-CSF and TNF. Though both subsets can induce naïve CD4+ T-cell proliferation, only interstitial DCs produce IL-10 and can induce the differentiation of naïve B cells into Ig-secreting plasma cells. Though no unique function has yet been formally attributed to LCs, there are hints they may be particularly efficient activators of cytotoxic CD8+ T cells. The majority of clinical studies to date have been carried out with ex vivo-generated monocyte-derived DCs, which resemble a single subset, i.e., interstitial DCs. Another example of DC subsets is evident in fresh human blood, each representing a small fraction of the mononuclear cells. One terminology for these subsets is CD11c+ myeloid DCs, and CD11c– plasmacytoid DCs. The CD11c+ cells are thought to be similar to monocyte derived DCs, whereas the CD11c– plasmacytoid cells are distinct in their capacity to make very large amounts of IFN- α , but much lower amounts of IL-12. The efficacy of these distinct human DC subsets will need to be compared in clinical studies.

5.2. The Optimal DC Maturation State and Stimulus

Immature DCs are weak immunogens. It has been reported that intranodal injection of immature DCs does not lead to significant immune responses, contrary to the intranodal injection of mature DCs in the same patient (67). Immature DCs can also be tolerogenic. Indeed, injection of immature DCs in healthy volunteers leads to the inhibition of CD8+ T-cell immunity to viral peptide with the appearance of peptide-specific IL-10-producing T cells (68,69). Presence of peptide-specific CD8+ regulatory T cells could be demonstrated in these cultures (68). In contrast, mature DCs (triggered, for instance, by a mix of cytokines such as IL-1 β /IL-6/TNF α /PGE2) induce functionally superior CD8+ T cells and polarize CD4+ T cells toward IFN- γ production (22,23). Thus, DC maturation is a critical parameter for the use of these cells in active immunization of patients. The nature and timing of the maturation stimulus may also be important as it may lead to a distinct pattern of gene expression in maturing DCs (70). It will be important, therefore, to identify stimuli that trigger an equally effective maturation program in the various human DC subsets, including those mobilized in vivo (71).

5.3. DC Dose, Frequency, Route, and Schedule of Injections

In most studies thus far, the DCs were usually given at 2- to 4-wk intervals, and at doses between 4 and 40 million without striking differences in results. In vitro studies on human T-cell activation by DCs would predict that higher doses of DCs given more frequently should provide more intense and durable TCR triggering and thus promote T-cell priming and polarization. However, frequent stimulation might also cause activation-induced death of T cells. Also, the induced CTLs may kill booster DCs and thus reduce efficacy (72). Yet, fully mature DCs seem resistant to CTL lysis (73). Other important factors are the route of injection and the migration of DCs from the injection site (74). Though

antigen-loaded DCs may prime T-cell responses regardless of the route of injection, the quality of responses may be affected by it (75,76).

A question that has yet to be addressed is the duration of DC-based vaccination against cancer. How long shall we keep immunizing patients in whom vaccine induces immune responses and disease stabilization? Although current data do not permit a conclusion, it is possible that consolidation vaccine therapy may need to be lifelong, and may require adjunctive immune therapies such as cytokines to support T-cell memory.

5.4. Source, Preparation, and Antigen-Loading Strategy

Several systems have been employed to load DCs with TAA. Loading MHC class I molecules with peptides derived from defined antigens is most commonly used, and is also applied to recently identified MHC class II helper epitopes. Although important for “proof of concept” studies, the use of peptides is limited because of their restriction to a given HLA type, the limited number of defined TAA, and the induction of a restricted repertoire of T-cell clones. Furthermore, quantity and longevity of peptide loading is difficult to control. Alternative strategies that provide both MHC class I and class II epitopes and lead to a diverse immune response involving many clones of CD4+ T cells and CTLs are needed. These include: recombinant proteins, exosomes (vesicles rich in MHC-peptide complexes and heat shock proteins) (77), viral vectors, plasmid DNA, RNA transfection (78), dying tumor cells, opsonized tumors (38), immune complexes, or fusion of tumor cells and DCs (79). Few comparative data presently exist between these strategies. Tumor-derived RNA may be an attractive source of patient-specific tumor antigen, as it can be adapted to small amounts of tumor cells as pioneered by the group at Duke (78,80–82). Whole tumor cell-based approaches have the theoretical advantage of immunizing against multiple epitopes from tumors, including subdominant or cryptic epitopes. A potential concern, however, is with the induction of autoimmunity by eliciting a response to self-antigens, particularly with repeated injections (83). On the other hand, complex antigenic mixtures derived from autologous tumor cells (e.g., opsonized cells or RNA) may represent the optimal antigens, as true tumor rejection antigens may be specific for each patient’s tumor. Indeed, our recent studies in myeloma suggest that T-cell reactivity against autologous tumor is largely patient specific, and most of these T cells do not recognize epitopes from allogeneic tumor, tumor cell lines, or nonneoplastic lymphoid cells (47).

6. CONCLUSIONS

In conclusion, DCs are an attractive target for therapeutic manipulation of the human immune system and to enhance immune responses in cancer (8,9). Immunization with ex vivo-generated DCs has proven feasible, and permits the enhancement as well as the dampening of antigen-specific immune responses in man (22,23,68,69). These ex vivo strategies should help identify the optimal parameters for DC targeting in vivo. Targeting DCs either in vivo or ex vivo, for antigen-specific immune modulation may therefore be of value for not only cancer and infectious disease, but also autoimmunity and organ transplantation. However, DC vaccination is at an early stage of clinical development, and many aspects of DC biology need to be carefully studied, to facilitate optimal application of these adjuvants in the clinic.

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Exosomes for Immunotherapy of Cancer

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1. EXOSOME BIOGENESIS AND COMPOSITION

The biology of small vesicles (1–4) secreted from antigen-presenting cells (APCs) recently raised a great deal of interest with the demonstration of their potent immunostimulatory functions in tumor models (5,6). The origin of vesicle secretion was first described (7) in differentiating red blood cells where multivesicular bodies (MVBs) fused with plasma membrane in an exocytic manner. This exocytic pathway was later shown to occur in a wide variety of cell types such as B lymphocytes, mastocytes, immature dendritic cells (DCs), platelets, cytotoxic T lymphocytes (CTLs), fibroblasts, epithelial cells, and tumor cells (6,8–15). Vesicles exocytosed from MVBs into the extracellular medium are referred to as “exosomes” and should not be confused with the more recently described “ribonuclease complex” also named exosome.

Exosomes are unilamellar vesicles of 50–100 nm diameter forming by inward budding of the endosomal membrane in a process that sequesters particular proteins and lipids (9). The machinery that drives MVB formation is directly relevant to exosome production. Only recently, a few aspects of the responsible mechanisms have been unraveled. In yeast, at least 15 class E VPS genes are required for protein sorting into MVBs and those have orthologs in mammalian cells (16). The MVB pathway in yeast and mammals requires phosphatidyl-inositol (PI) 3-kinase and PI(3)P5-kinase activities (17). Hrs is a PI (3)P-binding protein playing a critical role in MVB formation (18) by recruiting clathrin to endosomes and downregulating EGF-R through interaction with sorting nexin

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1 (19). In yeast, ubiquitination of endosomal cargo serves as a signal for sorting in MVBs. Not all proteins require ubiquitination for their sorting into the MVB pathway. Tetraspanins may partition into raft-like microdomains (20) and associate with integrins, major histocompatibility complex (MHC) class II, CD86 growth factor receptors, providing a mean to target proteins into the MVB pathway. When endocytosed proteins that normally recycle are aggregated by specific antibodies, they are mistargeted into the MVB pathway and subsequently secreted within exosomes (21,22).

In fibroblasts, the secretion of lysosomal contents is Ca⁺⁺ dependent and regulated by syntaxin VII. Though exosome secretion appears to be constitutive in immature DCs, it may proceed according to the same principles. In mast cells, IgE-antigen complexes and increased cytosolic Ca⁺⁺ triggers exosome release. In T lymphocytes, fusion of MVBs with plasma membrane may also be stimulated through increased cytosolic Ca⁺⁺ (23,24).

Protein composition of exosomes resembles that of MVBs' internal vesicles and has been characterized using immuno-blotting (9), peptide mass spectroscopy mapping (25,26), and affinity extraction onto magnetic beads followed by phenotyping by flow cytometry (27). The unique composition of exosomes may confer specific functions to such secreted vesicles. Exosomal composition may vary depending on the cells from which they originate. For example, exosomes from reticulocytes are enriched in TfR, exosomes from CTL transfer perforin, and granzymes and exosomes from platelets exhibit Von Willebrand factor. The common proteins include chaperones such as Hsc73 and Hsc90, subunits of trimeric G proteins, Tsg101, cytoskeletal proteins, and tetraspanins (CD9, CD63, CD81, CD82). Importantly, exosomes secreted from APCs, i.e., B cells, DCs, lymphocytes, mast cells, and epithelial cells are bearing MHC class II and I molecules. Tumor cell-derived exosomes also harbor whole cytosolic tumor antigens (such as TRP, gp100, Mart1 in melanoma, and Her2/Neu, EGF-R in ovarian or breast cancers [4], actinin-4 in mesothelioma [Hegmans, in preparation]).

Interestingly, DC-derived exosomes bear specific proteins (CD9, CD11b, lactadherin) that might be involved in exosome targeting *in vivo*.

Sorting behavior of lipids in MVBs is determined by the nature of their hydrophobic tails. Lysobisphosphatidic acid is incorporated into MVBs' internal vesicles and found on exosomes. Stoorvogel et al. described the lipid composition of B-cell-derived exosomes and found enrichment in cholesterol and sphingomyelin (28).

2. PRODUCTION AND PURIFICATION OF EXOSOMES

Since exosomes mediate MHC class I- and class II-restricted T-cell stimulatory capacity (29–31) and efficiently substitute for whole DC cultures (5), good manufacturing process (GMP) laboratory procedures for exosome harvesting and purification have been set up for clinical implementations (32). Exosomes derived from DC and tumor cell culture supernatants can be readily purified within 4–5 h starting from 2–3 liters of culture supernatant based on their physical properties. Exosomes float on sucrose gradients at a density ranging from 1.13 (for B-cell-derived exosomes) to 1.210 g/cm³ (for dexosomes/texosomes/texas) (4,6,11,25,33,34). Ultrafiltration of the clarified culture supernatant through a 500-kDa hollow fiber membrane followed by ultracentrifugation onto a 30% sucrose/deuterium oxide cushion (density: 1.13–1.210 g/cm³) reduced the volume and protein concentration approx 200-fold and 1000-fold, respectively. The percentage recovery of exosomes is about 40–50%, based on the exosomal MHC class

II concentration of the starting clarified supernatant. This methodology was extended to a miniscale process with comparable results. Likewise, the classical sedimentation technique is a tedious and less productive process carrying over along with exosomes contaminants of the culture medium. Furthermore, the development of quality control assays allowed qualitative and quantitative standardization of exosome preparations. Immunocapture assays assessing exosomal contents in MHC class I and II molecules as well as FACS determination of exosomal protein patterns after coupling onto macroscopic beads are currently used to calibrate exosome dosages in the first phase I trial (24). Exosomes are secreted from monocyte-differentiating DCs from Day 5 to Day 6 using culture conditions devoid of maturing agents (granulocyte-macrophage colony-stimulating factor [GM-CSF]/interleukin-4 [IL-4]). The amounts of exosomal MHC class I molecules recovered in a 24-h DC culture supernatant using such a purification process were evaluated. From $2 \cdot 10^{11}$ to 10^{12} exosomal MHC class I molecules were found in the pellets of $3 \cdot 10^7$ – 10^8 immature human DC culture/24 h. From 10^{14} to 10^{15} exosomal MHC class II molecules were recovered from GMP human DC cultures (5×10^8 – 10^9 cells) derived from a single leukopheresis in metastatic melanoma patients (35).

3. BIOLOGICAL EFFECTS OF EXOSOMES

The primary aim of exosomal release for a cell might be to discard membrane proteins. This role was suggested for reticulocyte-derived exosomes that carry transferring receptors (TfRs) useless in erythrocytes (36). Thus, exosomes could be an alternative to lysosomal degradation, for example, to eliminate proteins that resist degradation by lysosomal proteases.

However, based on their protein composition, i.e., enrichment for MHC complexes, hsp, and some targeting molecules, it is conceivable that exosomes and more specially APC-derived exosomes might be involved in amplification of antigen presentation. Raposo and colleagues (33) first observed that MHC class II molecules borne on Epstein-Barr virus (EBV)-transformed B-lymphocyte-derived exosomes were functional when associated with antigenic peptides. These exosomes could induce significant but weak MHC class II-restricted-CD4+ T-cell proliferation in vitro. We further demonstrated that the MHC class I/peptide complexes harbored by mouse and human DC-derived exosomes are functional to trigger peptide-specific, MHC class I-restricted T-cell clones and to elicit primary Tc1 lymphocyte responses in vitro and in vivo. However, exosome-mediated T-cell responses require DC in vitro and in vivo for both CD4+ and CD8+ T-cell priming (29–31). Moreover, tumor peptide-pulsed DC-derived exosomes induce tumor growth retardation of established murine tumors in a T-cell-dependent manner (5).

In addition to transferring preprocessed antigens in MHC molecules, exosomes might also transfer hsp-associated peptides or cytosolic whole candidate tumor antigens. We could demonstrate that melanoma cells release exosomes containing whole tumor proteins such as MART-1/melanA. Following DC uptake of such melanoma-derived exosomes, cross-presentation of MART-1 peptides by DC MHC class I molecules could be observed. In vivo, DCs pulsed with texosomes induced potent CD8+ T-cell-dependent antitumor effects against established mouse tumors (6).

The relevance of exosome secretion by an immature DC remains questionable. Since exosomes are specifically enriched with functional MHC complexes and bear targeting molecules for DCs or other APCs, and since exosome uptake by recipient DCs appears

rapidly saturated (our unpublished data), it is tempting to speculate that secretion of exosomes by DCs is an amplification pathway for the antigen presentation network, enabling rapid dissemination of processed MHC complexes for T-cell stimulation. As suggested for the immunological synapse and for the tetraspan-enriched domains (37), the presence of exosomes on the membrane of immature DCs might trigger sustained engagement of T-cell receptors. One could postulate that exosome secretion might occur in the lymph or in the T-cell area of lymphoid organs.

Likewise, it is also conceivable that exosomes are involved in peripheral tolerance and inhibition of immune responses. Experiments in rats reported that intraperitoneal injections of exosomes derived from a gut epithelial cell line loaded with Ag led to a decrease of the Ag specific delayed-type hypersensitivity reaction (38). Moreover, in allogeneic cardiac transplantation models, Cuturi and colleagues showed that donor LEW.1W DC exosomes injected intravenously induce LEW.1W heart allograft tolerance in LEW.1A rat recipients. Prophylactic usage of exosomes in recipients was more efficient in prolonging allograft survival than therapeutic usage (39). Finally, recent studies showed that T cells can acquire peptide/MHC class II complexes from APCs (10,13). These T lymphocytes were killed by neighboring lymphocytes, suggesting that fratricidal T-cell killing could represent a negative feedback loop of immune responses (40). The role of exosomes in such a membrane exchange has not been investigated.

All reported studies dealing with exosome functions utilized exosomes from culture supernatants of propagated cell lines. Exosomes have been observed *in vivo* for the first time in electron microscopy studies of human follicular DCs (FDCs) in tonsils (2). Forty- to 70-nm microvesicles expressing tetraspanins have been observed *in situ* on the plasma membrane of FDCs. They also expressed MHC class II molecules that are not neo-synthesized by FDCs. These vesicles contained lysobiphosphatidic acids, a lipid found on the luminal vesicles of MVBs. The possibility that these vesicles could be iccosomes, i.e., interconnected immune complex-coated body (antigen–antibody complexes) was excluded on the basis of their size (0.3–0.7 μm) and composition (flocculent peroxidase-positive material). The authors hypothesized that these microvesicles were exosomes originating from germinal center B lymphocytes or neighboring DCs.

We recently reported that high amounts of tumor exosomes accumulate in the malignant effusions of patients bearing different types of tumors (melanoma, breast, lung, ovarian cancer, mesothelioma) (4). Exosomes harvested from ascitis of melanoma patients efficiently transport MART-1 tumor antigen to monocyte-derived dendritic cells (MD-DCs) for cross-presentation to MART-1-specific CTL clones. Moreover, in vitro stimulation assays aimed at priming peripheral CD8+ T lymphocytes using autologous DCs, ascitis-derived exosomes could prime naïve T cells recognizing the autologous primary tumor culture in an MHC class I-restricted manner in seven out of nine cancer patients. Whether exosomes produced *in vivo* mediate any immune functions remains hypothetical. The poor immunogenicity of texosomes *in vivo* might be a result of the absence of myeloid DCs and/or the presence of immunosuppressive agents (i.e., IL-10 and transforming growth factor-β [TGF-β]) in ascitis.

Altogether, these data suggest that exosomes constitute a novel pathway of antigen transfer-modulating T-cell activation, and that such antigen-presenting vesicles could be suitable for cancer immunotherapy. The mechanisms of their *in vivo* functions and their relevance remain to be established.

4. EXOSOMES AND THEIR POTENTIAL APPLICATION IN IMMUNOTHERAPY

Active immunotherapy with mature DCs is being extensively tested for treatment of malignancies. DC-based immunotherapy, however, remains difficult to handle for scale up, definition of quality control parameters, and long-term storage. Indeed, many investigators have shown that one leukopheresis enables up to 4–10 DC injections, whereas there is evidence that a larger number of injections, perhaps spanning over a year or more, appear to be required for long-lasting antitumor protections. Maturation of DCs has been shown to be necessary for efficient DC migration and Tc1 differentiation in lymph nodes. However, GMP cytokine cocktails, required for efficient maturation of DCs need to be, more broadly defined. Finally, regulatory authorities' criteria for the development of therapeutics require biochemical or functional characterization of off-the-shelf products that cannot be readily achieved.

Exosomes might be helpful to overcome part of the shortcomings related to technical development of DCs. Exosome composition can be defined, MHC class I and II content can be measured, and exosomal membranes are stable and can be stored for at least 6 mo. Preclinical studies aimed at demonstrating exosome immunogenicity *in vitro* and *in vivo* will be presented as well as the pioneering phase I clinical trial.

4.1. DC-Derived Exosomes Mediate T-Cell-Dependent Tumor Rejection in Mice

The original observation was that exosomes secreted from bone marrow DC pulsed with acid-eluted tumor peptides elicited T-cell dependent tumor rejection in P815-established tumors and significant tumor growth retardation in TS/A mammary tumors. Exosome-mediated tumor rejection was tumor peptide-specific and long-term protection was tumor-specific. In tumor-free mice, tumor-specific CTLs could be recovered after *in vitro* restimulation of splenocytes (5). The precise mechanisms accounting for the immunogenicity of DC exosomes is being extensively investigated.

4.2. DC-Derived Exosomes Bear Functional MHC Class I and Class II/Peptide Complexes

Exosomal MHC class I molecules can be efficiently loaded directly, after acid elution of purified exosomes (32; Dee Shu, unpublished data). In contrast, exosomal MHC class II molecules can present peptides that have been pulsed onto whole DC culture (25,26; Dee Shu, unpublished data). We are currently reporting that MHC class I and II/peptide complexes harbored by mouse or human exosomes activate specific CD8+ or CD4+ T lymphocytes but require DCs (29,31). Exosomal HLA-A2/Mart1 peptide complexes trigger specific HLA-A2-restricted, Mart1-specific CTL clones when pulsed onto DC *in vitro* (30). In the absence of DC, exosomes do not directly stimulate T cells *in vitro*. In the HHD2 HLA-A2 transgenic mouse model, inoculation of DC-derived exosomes bearing functional A2/MART-1 complexes (up to 10^{10} class I molecules) pulsed onto H-2b mature DCs allows expansion of specific A2/MART-1_{26–35} tetramer-positive CD8+ T cells in draining lymph nodes (Fig. 1).

4.3. Phase I Trial Using Human DC-Derived Exosomes

These data were the rationale for launching vaccination with DC-derived exosomes in metastatic tumor-bearing patients. A GMP has been set up to harvest large amounts of MD-DC-derived exosomes (ultrafiltration followed by ultracentrifugation on a high-density sucrose/D₂O cushion) and load exosomal MHC class I molecules with synthetic tumor peptides. Phenotypical analysis of clinical-grade exosomes is allowed by FACS-beads-assay, i.e., DC-derived exosomes, are fixed onto 4.5-micron beads stained using surface antigen-recognizing fluorescent antibodies. An immunocapture assay determines the quantity of MHC class I and/or II molecules in each individual exosome batch. A feasibility and safety phase I study was undertaken in France (Institut Gustave Roussy and Institut Curie with Anosys Inc. biotechnological partnership) in HLA-A1/B35 and -DP04 patients bearing stage III/IV melanoma expressing MAGE-3. Dexosomes were purified from the culture supernatant of day 7 autologous MD-DCs. MAGE-3 peptides were loaded onto MD-DCs (in the first six patients), or directly onto dexosomes (in nine patients). Escalating doses of cryopreserved dexosomes were administrated by four weekly sc/id injections, then every 3 wk in patients who achieved stable disease or a tumor regression. Fifteen patients undergoing progressive disease have entered the study (metastatic sites including skin, nodes, lung, and liver). Feasibility of exosome harvesting was about 100% with a yield of exosome production allowing from 6 to 120 vaccine administrations. The vaccine therapy was well tolerated, without any evidence of Grade 2 toxicity. In indirect loading process, one stage III patient achieved stabilization and received eight additional vaccine injections with continual stable disease. Using direct loading but low exosome dosage, a second patient achieved a mixed response with regression of subcutaneous sites but lung progression. A third patient exhibited partial response at node sites. Briefly, three of six patients who received the highest dosage of directly loaded DEX exhibited objective responses (skin and lymph node lesions). It is noteworthy that two patients previously progressing with ALVAC-MAGE3A1 and MAGE3 protein were responding to exosomes. A case of antigen spreading and MHC class I loss variant was documented following exosome administration. The MAGE3-specific CTL responses detectable using specific fluorescent tetramers highlighted monoclonal responses in three patients.

Altogether, exosomes may represent a valuable alternative to boost immunological responses elicited with DCs or to substitute for DCs in immunotherapy of cancer.

4.4. Tumor-Derived Exosomes Mediate Tumor Antigen Cross-Presentation by DCs to T Cells

Ideally, immunotherapy strategies aimed at immunizing the host should be able to elicit T-cell-based immune responses directed against a broad repertoire of tumor rejection antigens. Though mature DCs appear to be the most potent natural adjuvants, suitable methods allowing efficient antigen uptake, processing, and presentation by DCs are still being developed. Several approaches involving the use of whole-tumor RNA, tumor lysates, apoptotic or necrotic debris, and fusion are currently under investigation. Wolfers et al. reported that (a) melanoma line-derived exosomes contain differentiation tumor antigens, (b) those tumor exosomes loaded onto DCs transfer shared tumor antigens

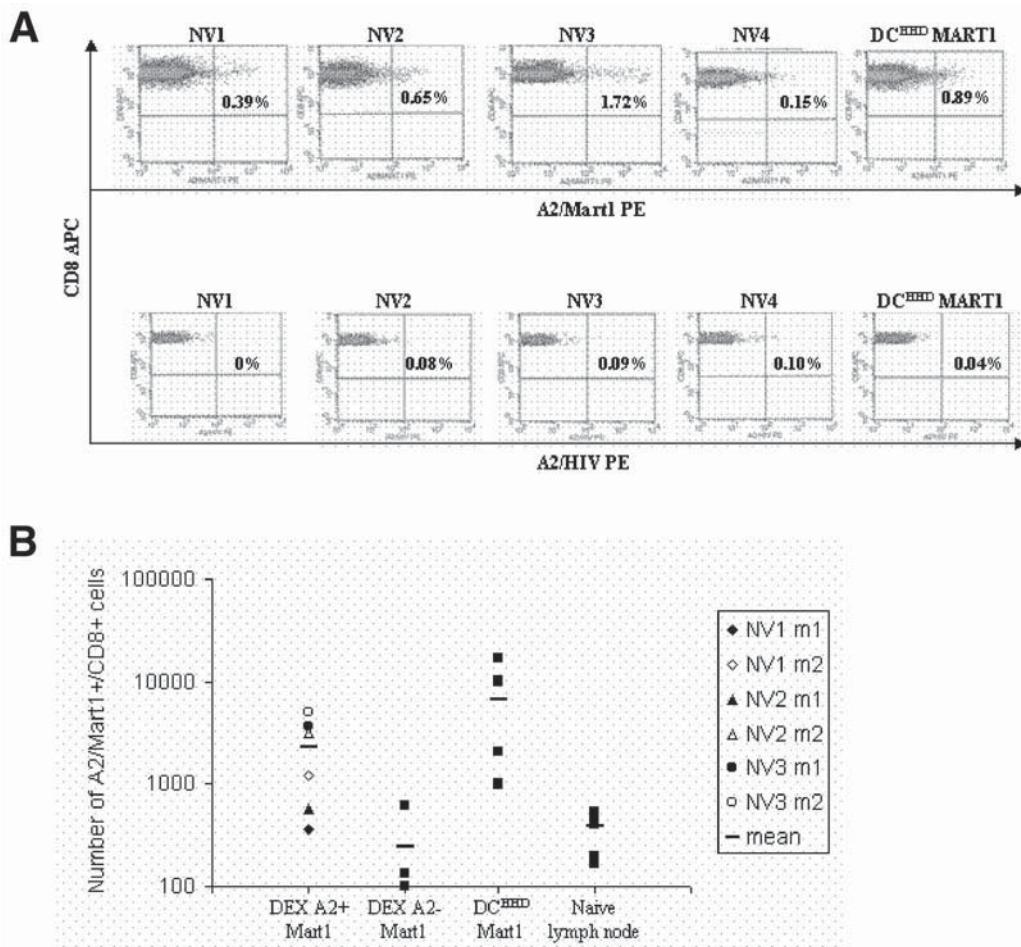


Fig. 1. Human exosomes presenting A2/Mart1 complexes are immunogenic in HHD2 transgenic mice. DEX A2⁺/Mart1 from normal volunteers (NV1, NV2, NV3) and DEX A2⁻/Mart1 from NV4 were inoculated in the footpad of mice, 6 d prior to lymph node harvesting. DEX/Mart1 were incubated with mature H2^b bone marrow-derived DCs prior to intradermal inoculation. In order to avoid nonspecific effects mediated by free peptide carryover, we used BM-DCs from H2^b wild type mice instead of HHD2 mice. Mature HHD2-derived BM-DCs pulsed with 10 μ M Mart1 peptides were used as positive controls of T-cell priming. Draining lymph node (LN) resident CD8⁺ T cells were examined using A2/Mart1 tetramers in all conditions and A2/HIV tetramers as irrelevant negative tetramers control.

Whereas the mean percentage and absolute number of CD3+/CD8⁺ T cells stained with A2/Mart1 tetramer in nonimmunized controlateral LNs was <0.2% (data not shown) and <2–5 \times 10² cells, respectively, the yields obtained in previous study with 3 \times 10⁵ mature unpulsed DCs was about 0.2% (data not shown).

In this setting, mature DCs pulsed with the relevant Mart1 epitope (DC^{HHD}/Mart1) allowed expansion of five times more A2/Mart1-specific CD3+/CD8⁺ T cells (0.9%, 1 \times 10³ cells).

Though DEX A2⁻/Mart1 exosomes from NV4 did not expand A2/Mart1 tetramer-binding CD8⁺ T cells (0.15%, 1 \times 10² cells), DEX A2⁺/Mart1 exosomes could allow significant expansion of A2/Mart1-binding CTLs in four of six mice immunized (NV1 S2, NV2 S2, NV3 S1, and NV3 S2; Fig. 1B), in comparison with the positive control DC^{HHD}/Mart1.

Altogether, exosomes bear MHC class I/peptide complexes that can be transferred to DCs to promote expansion of class I restricted-CTLs *in vivo*.

triggering MHC class I-restricted T clones in vitro, and (c) tumor exosomes are a source of rejection tumor antigens since tumor exosomes promote T-cell-dependent cross-protection against syngeneic and allogeneic tumors in mice (6).

4.5. Immunogenicity of Peritoneal Ascitis-Derived Exosomes

Tumor-derived exosomes are not simply released in vitro by tumor cell lines in culture supernatants. We examined malignant effusions for the presence of tumor-derived exosomes and analyzed exosome immunogenicity on autologous peripheral T lymphocytes. Ultracentrifugation on sucrose and D₂O gradients of 11 malignant effusions allowed isolation of abundant amounts of exosomes. Malignant effusions accumulate high amounts of membrane vesicles with a mean diameter of 60–90 nm. These vesicles bear antigen-presenting molecules (MHC class I ± MHC class II, heat shock proteins), tetraspanins (CD81), and contain tumor antigens (Her2/Neu, MART-1, TRP, gp100). Up to 2×10^{14} exosome-associated MHC class I molecules are recovered from malignant ascitis of 2–3 liters. Exosomes from melanoma patients shuttle MART-1 tumor antigen to MD-DCs for cross-presentation to MART-1-specific CTL clones. In seven of nine cancer patients, tumor-specific lymphocytes could be efficiently expanded from peripheral blood cells using autologous MD-DCs pulsed with autologous ascitis-derived exosomes (4). Therefore, tumor-derived exosomes accumulate abundantly in cancer patients. Ascitis exosomes represent a natural and novel source of tumor rejection antigens, opening up novel immunization avenues in advanced ovarian cancers or mesothelioma. The clinical implementations of ascitis-derived exosomes require the demonstration that exosomes from ascitis fluids can be immunogenic in mice leading to tumor rejection, and should be safe in preclinical GMP settings in rodents. We are also currently developing a clinical-grade process for purification of ascitis exosomes.

5. CONCLUSION

The purification process used for exosome isolation from DC cultures and ascites fluid allows harvesting of reproducible yields of 40- to 100-nm vesicles that are highly immunogenic in vitro. Exosomes are currently defined by their morphology (electronic microscopy), by their physical properties (stable at high temperature, floating at a density of 1.13–1.210 g/mL in a sucrose/D₂O gradient), by their proteic patterns (endocytic markers, i.e., tetraspanins and hsp73), and by their enrichment in MHC class I, II, CD86 molecules when derived from DCs. Importantly, exosomes efficiently transfer antigens from APC to APC, allowing initiation and amplification of antigen-specific immune responses. Immunization of melanoma patients with DC-derived exosomes was associated with objective clinical responses and prompted the initiation of phase II trials. However, the physiological relevance of exosomes remains to be established and might ensue from the identification of the critical components of their membrane accounting for antigenic transfer.

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Immunocytokines for Cancer Immunotherapy

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1. INTRODUCTION

Immunologic approaches to cancer therapy rely on two distinct capabilities of the immune system: targeting the tumor microenvironment by recognizing molecules expressed to a greater extent on tumor cells than normal cells and generating immune responses that can kill tumor cells (1,2). Immunocytokines, which are fusion proteins composed of a recombinant monoclonal antibody and a cytokine, capitalize on both of these capabilities by combining the ability of tumor-specific antibodies selectively to target tumors with the broad-based immunomodulatory activities of cytokines (3). This chapter describes the rationale for development of immunocytokines for cancer and discusses preclinical and clinical data on specific immunocytokines being investigated as potential cancer therapies.

2. IMMUNOCYTOKINES: RATIONALE AND HYPOTHESIZED MECHANISM OF ACTION

Immunocytokines combine the desired elements of two established modalities of immunotherapy: recombinant monoclonal antibody therapy and cytokine therapy.

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2.1. Monoclonal Antibodies for Cancer Therapy

The administration of recombinant monoclonal antibodies for cancer is based on the premise that tumor-specific antibodies can recognize and target cancer cells and, once in the tumor microenvironment, can inhibit tumor growth or induce tumor regression via mechanisms including antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (2,3). The anti-CD20 monoclonal antibody rituximab, for example, binds to the B-cell antigen CD20 expressed on B-cell lymphomas to kill cancer cells via ADCC and induction of apoptosis (4–6). The monoclonal antibody trastuzumab binds to the receptor for the growth factor HER-2, which is overexpressed in up to 30% of human breast cancers, to block the proliferative effects of this growth factor on breast cancer cells (7). Monoclonal antibodies have also been conjugated to tumor-killing substances such as radioisotopes, drugs, or toxins (8–10). Although monoclonal antibody therapy has yielded positive results in some human cancers, it has several limitations. Shortcomings include poor penetration of solid masses with resulting impaired distribution of antibody at the tumor, heterogeneity of antigen expression leading to suboptimal monoclonal binding to tumor, and limited direct activation of tumor cell destruction (2).

2.2. Cytokines for Cancer Therapy

Cytokines such as interferons and interleukins are administered for cancer because of their broad-based immunostimulatory effects including generation of tumor-reactive lymphocytes (11). Interleukin-2 (IL-2), or aldesleukin, which is indicated for the treatment of adults with metastatic renal cell carcinoma and melanoma, is the most studied cytokine. IL-2 dose-dependently activates cellular immunity and causes release of other immune-boosting cytokines *in vivo* (11). Systemic cytokine therapy is generally limited by rapid degradation and elimination of the cytokine, the inability to achieve optimal concentrations in the tumor microenvironment, and dose-dependent toxicity, including life-threatening side effects such as vascular leak syndrome and orthostatic hypotension (12–15). Cytokine gene therapy, in which a cytokine gene (such as that for IL-2) is introduced into tumor cells, is being explored to overcome some of these limitations of systemic cytokine administration. However, cytokine gene therapy is technologically challenging and resource-intensive.

2.3. Rationale for Immunocytokines

As fusion proteins combining monoclonal antibodies with cytokines, immunocytokines were developed to improve upon the benefit:risk ratios of monoclonal antibodies and cytokines alone or as combination therapy. Immunocytokines are hypothesized to confer two possible advantages over currently available immunotherapies (3). First, by providing both antibody effector activities and a cytokine cosignal for the generation of cytotoxic cellular immunity, immunocytokines may amplify antitumor immune responses relative to those obtained with current immunotherapies. Second, by targeting delivery of cytokines to the tumor microenvironment, immunocytokines deliver biologically active concentrations of cytokines at lower and less toxic doses than are required by systemic cytokine therapy. Data from *in vitro* studies and *in vivo* tumor models have borne out these hypotheses by showing that immunocytokines stimulate tumor-suppress-

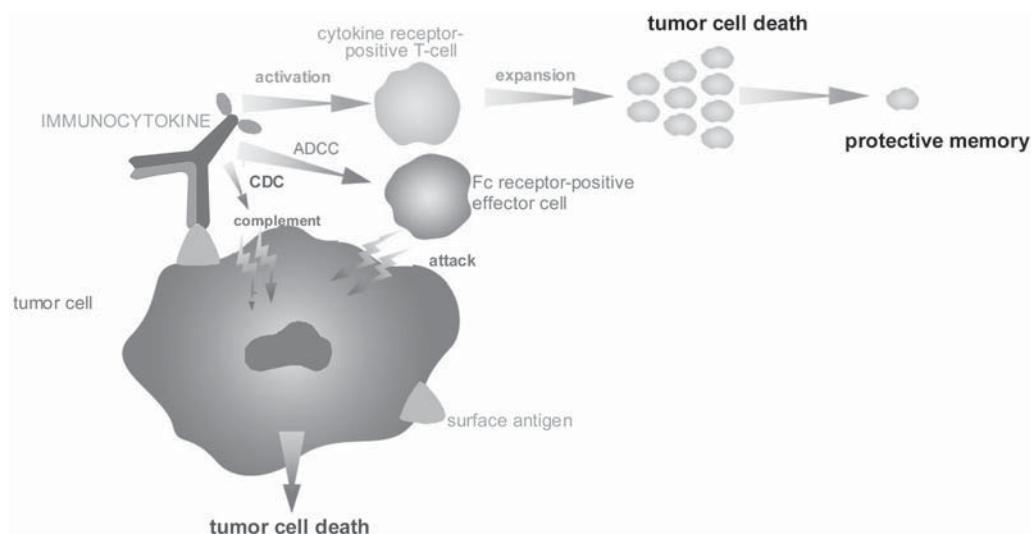


Fig. 1. Hypothesized mechanisms of antitumor action of immunocytokines.

ing immune responses and that in vivo administration of immunocytokines causes a greater antitumor effect than administration of a mixture of an equivalent dose of antibody and cytokine. Immunocytokines also appear to prolong cytokine biological activity relative to that of systemically administered cytokines.

2.4. Hypothesized Mechanisms of Action of Immunocytokines

The mechanisms of immunocytokine-mediated antitumor activity, like those of the antitumor activity of monoclonal antibodies or cytokines administered alone, are not fully understood but appear to be multifactorial (3,16,17). Once bound to its specific antigen on the tumor cell surface, the antibody portion of the immunocytokine is hypothesized to mediate both ADCC—which involves antibody activation of effector cells such as macrophages, granulocytes, monocytes, and some natural killer (NK) cells—and CDC, which involves activation of the complement cascade (Fig. 1). The cytokine portion of the immunocytokine is hypothesized to mediate a range of immunomodulatory responses in the tumor microenvironment by activating cells of the immune system through their cytokine receptors. The cytokine portion of the immunocytokine also provides a costimulatory signal for enhancing both NK-cell- and T-cell-mediated antitumor responses.

3. IMMUNOCYTOKINES IN DEVELOPMENT

Several immunocytokines are being explored for potential use in human cancers, and two (hu14.18-IL-2 and huKS-IL-2) have been studied in human cancer with promising results to date. Besides these IL-2-based immunocytokines, which have been thoroughly assessed in preclinical models and are undergoing evaluation in the clinic, IL-12- and granulocyte-macrophage colony-stimulating factor-based (GM-CSF-based) immunocytokines are being assessed in preclinical tumor models.

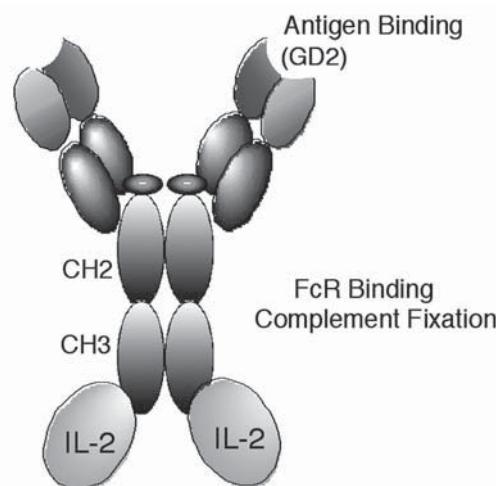


Fig. 2. Immunocytokine hu14.18-IL-2.

3.1. Anti-GD₂-IL-2 Immunocytokine

The anti-GD₂ immunocytokine hu14.18-IL-2 (EMD 273063) is currently in phase I clinical trials (18). This immunocytokine consists of recombinant human IL-2 and a humanized monoclonal antibody directed against human GD₂, which is a disialoganglioside antigen expressed by tumors of neuroectodermal origin including malignant melanoma, neuroblastoma, some sarcomas, and some small-cell lung carcinomas. Each immunocytokine molecule is composed of two cytokine molecules fused to each of the two heavy chains of a monoclonal antibody via a peptide linkage at the carboxyl terminus (Fig. 2).

3.1.1. BIOLOGICAL ACTIVITY

Preclinical studies with the anti-GD2-IL-2 fusion protein were conducted with a chimeric form (i.e., ch14.18-IL-2) as well as the humanized form that is EMD 273063 (i.e., hu14.18-IL-2). Early studies focused on establishing the biological functionality of the immunocytokine and on determining whether the fused protein retained full antibody and cytokine functions. That ch14.18-IL-2 retains its functionality as an antibody was confirmed by the finding that the immunocytokine and ch14.18 had comparable GD₂-binding patterns in direct binding assays with human neuroblastoma cells (19). Dissociation constants (K_d) for the immunocytokine and ch14.18 were 24 nM and 18 nM, respectively. A similar pattern of results was observed in separate studies with hu14.18-IL-2 (18). That ch14.18-IL-2 retained cytokine properties was confirmed by the finding that the fusion of ch14.18 with IL-2 did not reduce IL-2 activity relative to that of commercially available IL-2 in a standard proliferation assay involving either mouse or human T-cell lines (16). Furthermore, the ability of a melanoma-specific T-cell line derived from tumor-infiltrating lymphocytes (TIL) to kill autologous GD₂-positive melanoma tumor cells was enhanced when target cells were precoated with ch14.18-IL-2. This effect of the ch14.18-IL-2 exceeded that observed when these TILs were cultured with autologous tumor cells in higher concentrations of free IL-2 (16). In another study, human lymphocyte-activated killer cells created by incubating human peripheral blood

Table 1
Antitumor Activity of huKS-IL-2 in a Mouse Xenograft Model

<i>Group</i>	<i>Amount of lung surface covered</i>	
	<i>Mean</i>	<i>Range</i>
Without lymphocyte-activated killer cells PBS (control)	98%	95–100%
With lymphocyte-activated killer cells PBS (control)	95%	90–100%
hu14.18 16 µg + IL-2 16,000 U	90%	80–95%
hu14.18-IL-2 16 µg	0% ^a	0%
hu14.18-IL-2 8 µg	25% ^a	0–70%
hu14.18-IL-2 2 µg	78%	0–100%

From ref. 18.

^a $p < 0.005$ vs PBS control

mononuclear cells with recombinant human IL-2 showed enhanced ADCC when pre-treated with ch14.18-IL-2 immunocytokine (10 µg/mL containing 30,000 IU of recombinant human IL-2) compared with administration of either ch14.18 alone (10 µg/mL) or a mixture of ch14.18 (10 µg/mL) and 30,000 IU recombinant human IL-2 (19).

Analyses of serum from mice injected intravenously with ch14.18-IL-2 show that the half-life of the immunocytokine was approximately 4 h (20,21). This value is shorter than the half-life of the ch14.18 monoclonal antibody but substantially longer than the half-life of soluble IL-2.

3.1.2. ANTITUMOR ACTIVITY IN PRECLINICAL MODELS

The antitumor activity of the anti-GD₂-IL-2 immunocytokine was assessed in both xenograft models that studied effects on human tumor cells in SCID (severe combined immunodeficiency) mice reconstituted with human lymphokine-activated killer cells and in syngeneic transplantable murine tumor models that assessed antitumor effects in immunocompetent hosts (19,22,23). Investigations focused primarily on metastases of melanoma and neuroblastoma, both of which express the GD₂ antigen. These studies establish that the anti-GD₂-IL-2 fusion protein was more effective against intradermal tumors and metastatic tumors of the lung, liver, and bone marrow than mixtures of the antibody and cytokine or administration of these components alone. For example, immunocytokine ch14.18-IL-2 was more effective than ch14.18 alone or recombinant human IL-2 alone in suppressing hepatic neuroblastoma metastases in SCID mice reconstituted with human lymphokine-activated killer cells, and it doubled survival times relative to those observed with recombinant human IL-2 alone (19,23). Similarly, immunocytokine ch14.18-IL-2 administered 1, 4, or 8 d after tumor inoculation, but not treatment with a mixture of ch14.18 and IL-2, eliminated disseminated, established pulmonary and hepatic metastases in C.B-17 SCID/SCID mice reconstituted with human lymphokine-activated killer cells (24). Comparable results were obtained in a study with the humanized form of the immunocytokine (18). A dose of 16 µg for seven consecutive days prevented the growth of pulmonary metastases after intravenous infusion of the human melanoma line M24met into SCID mice whereas treatment with a mixture of the antibody and the cytokine was ineffective (Table 1; 18).

Table 2
Efficacy of ch14.18-IL-2 for Pulmonary Metastases Having Antigenic Heterogeneity

<i>Treatment^a</i>	<i>Tumor</i>	<i>Number of Foci^b</i>
Experiment 1		
None	B16	>500, >500, >500, >500, >500, >500, >500
rIL-2+ch14.18	B16	129, 145, >500 >500, >500, >500, >500
ch14.18-IL-2	B16	97, 138, >500, >500, >500, >500, >500
None	B78-D14 + B16	>500, >500, >500, >500, >500, >500, >500
rIL-2+ch14.18	B78-D14 + B16	104, 179, 189, >500, >500, >500, >500
ch14.18-IL-2	B78-D14 + B16	0, 0, 2, 7, 9, 12, 21, 43
Experiment 2		
None	B16	173, >500, >500, >500, >500, >500
ch14.18-IL-2	B16	158, >500, >500, >500, >500, >500, >500
None	B78-D14 + B16	>500, >500, >500, >500, >500, >500
ch14.18-IL-2	B78-D14 + B16	0, 0, 2, 5, 9, 12, 18, 29

Adapted with permission from 25.

^aAll experimental groups started with eight mice. Pulmonary metastases were induced by intravenous injection of 1×10^6 B16 cells (GD₂-negative) or the combination of 1×10^6 B16 and 5×10^6 B78-D14 (GD₂-positive) cells. One week postinoculation, 7 consecutive days of treatment with PBS (control), ch14.18 8 µg + 24,000 IU recombinant IL-2 (rIL-2), or ch14.18-IL-2 8 µg was initiated.

^bDifferences in numbers of metastatic foci between animals with mixed tumors treated with the immunocytokine and all control groups were statistically significant ($p \leq 0.002$).

Immunocytokine ch14.18-IL-2 was also effective against established subcutaneous tumors (~25 µL) in a syngeneic mouse tumor model when administered intravenously for 7 d beginning 10 d after inoculation of tumor cells (25). All animals exhibited objective tumor responses, with 37.5% having complete remission and 62.5% having partial remission. The immunocytokine induced partial responses and delayed tumor growth even when treatment was withheld until 35 d after tumor inoculation so that large subcutaneous tumors (~1 cc) were established.

Another study using a syngeneic mouse tumor model demonstrates that immunocytokine therapy is effective even for tumors with heterogeneous expression of the target antigen—traditionally an obstacle for antibody-based immunotherapy (25). Pulmonary metastases heterogeneous with respect to GD₂ expression were induced by intravenous injection of GD₂-positive and -negative B16 melanoma cells into mice at a ratio of 5 to 1. Treatment with ch14.18-IL-2, but not with a mixture of the antibody and the cytokine, significantly reduced the number of metastases in 62.5% of animals and resulted in complete cure in 37.5% of the animals (Table 2; 25). The immunocytokine was ineffective in animals inoculated with only GD₂-negative cells. The authors attributed the anti-tumor effect to the specific cellular immune response elicited by the anti-GD₂-IL-2 fusion protein. It is hypothesized that, by delivering effective cytokine concentrations to the tumor microenvironment, immunocytokine therapy can induce T- and NK-cell-mediated immune responses that operate independently of the GD₂ target antigen (3).

3.1.3. IMMUNE MECHANISMS OF THE ANTI-GD₂-IL-2 IMMUNOCYTOKINE

The precise mechanisms of the antitumor effects of the anti-GD₂-IL-2 immunocytokine have not been definitively established. Several lines of evidence show that T

cell-mediated as well as NK-cell-mediated immune mechanisms are involved in the antitumor effect, with different patterns seen for different tumor models. The T-cell dependence of the efficacy of ch14.18-IL-2 in the B16-derived murine melanoma model is suggested by the finding that ch14.18-IL-2 was unsuccessful in eliminating pulmonary melanoma metastases in C57BL6 SCID/SCID mice, which lack functional T cells, but was successful in beige/beige mice, which have functional T cells and lack functional NK cells (3). Specific involvement of CD8+ T cells is consistent with the observations that lymphocytic infiltrate from subcutaneous tumor biopsies obtained after immunocytokine treatment contained primarily CD8+ T cells (25) and that immuno-cytokine eradication of pulmonary melanoma metastases was unsuccessful in CD8+-depleted beige/beige mice treated with ch14.18-IL-2 (3) or CD8+-depleted immunocompetent C57BL6J mice treated with hu14.18-IL-2 (26). Furthermore, CD8+ cells isolated from spleens of ch14.18-IL-2-treated mice demonstrated major histocompatibility complex (MHC) class I-restricted cytotoxicity *in vitro* to cells from the same tumor (27). Research with hu14.18-IL-2 suggests that the CD8+-mediated antitumor response is augmented by help from CD4+ cells as the antitumor response to the immunocytokine was reduced in mice lacking CD4+ cells (26).

Additional support for a T-cell-mediated mechanism of the antitumor effects of ch14.18-IL-2 comes from the demonstration of durable and transferable tumor immunity after administration of the immunocytokine (28). C57BL6 mice with pulmonary metastases induced by intravenous injection of syngeneic B78-D14 murine melanoma cells were treated 1 wk postinoculation with a 7-d course of ch14.18-IL-2. Six wk postinoculation, mice were rechallenged with intravenous injection of B78-D14 cells. The previous experience with the immunocytokine compared with no treatment or treatment with an antibody–cytokine mixture resulted in complete protection against tumor challenge in 7 of 12 mice, and tumor growth was significantly reduced in the remaining 5. The specificity of this antitumor effect was demonstrated by the finding that mice protected against B78-D14 tumors were not also protected against tumors induced by intravenous injection of EL-4 thymoma cells, which are syngeneic with C57BL6 mice and, like B78-D14 cells, express the GD₂ antigen. Thus, protective immunity induced by ch14.18-IL-2 is at least in part independent of the GD₂ antigen that is used as the docking site for delivery of IL-2 to the tumor microenvironment.

Besides being durable within animals, the tumor-protective immunity conferred by ch14.18-IL-2 was transferable between animals (28). Lymphocytes from spleens of mice successfully protected from B78-D14 tumors by ch14.18-IL-2 were transferred to naïve C57BL6 SCID/SCID mice 24 h before they were challenged with B78-D14 tumor cells. Of eight mice studied, five were completely protected from development of tumors, and three showed inhibited tumor growth relative to mice given lymphocytes from naïve mice or from mice with an untreated subcutaneous tumor. Considered in aggregate, the data in mouse B78-D14 melanoma models establish that the anti-GD₂-IL-2 immunocytokine induces T-cell-mediated immune responses that can mediate antitumor effects including durable, transferable protective immunity. In this model, the ch14.18 IL-2 molecule appears to localize to the *in vivo* sites of GD₂-positive tumors and to induce a T-cell response to tumor antigens, including those other than GD₂.

Whereas mouse B78 melanoma studies establish an important role of T-cell-mediated immune responses in anti-GD₂-IL-2 immunocytokine effects, mouse neuroblastoma

models establish an important role of natural killer cell-mediated immune responses. In the NXS2 syngeneic neuroblastoma model, ch14.18-IL-2 was effective at inhibiting tumor growth in SCID/SCID mice lacking T cells or in immunocompetent mice depleted of CD8+ cells but was ineffective in SCID/beige mice lacking both T cells and NK cells or immunocompetent mice depleted of NK cells (3). However, ch14.18-IL-2 demonstrated antitumor efficacy among SCID/beige mice injected with NK cells. These *in vivo* data are consistent with *in vitro* work demonstrating ch14.18-IL-2-associated increases in NK cell activation and with immunohistological evidence of positive staining for NK cells in liver biopsies of tumor-inoculated mice that had been successfully treated with immunocytokine. Together, these findings and more recent data (29) suggest that a NK-cell-mediated mechanism may be responsible for the antitumor effects of ch14.18-IL-2 in the NXS2 neuroblastoma model.

3.1.4. CLINICAL DATA: MELANOMA

Immunocytokine hu14.18-IL-2 is currently being evaluated in phase I studies as an immunotherapy for tumors of neuroectodermal origin. A recently completed phase I trial assessed the safety, toxicity, and immunologic effects of hu14.18-IL-2 in adult subjects with metastatic melanoma (18,30–32). Immunocytokine hu14.18-IL-2 was administered as a 4-h intravenous infusion (0.8, 1.6, 3.2, 4.8, 6.0, or 7.5 mg/m²/d) on d 1, 2, and 3 of week 1; and subjects with regression or stable disease could receive a second course of therapy on week 5. Thirty-three subjects with advanced melanoma were enrolled. Of 32 subjects who completed the first course of therapy, 18 had stable disease and completed the second course of therapy, at the end of which 8 subjects had stable disease (32). No Grade 4 adverse events were observed. Grade 2 adverse events included transient fever, hypotension, chills/rigors, arthralgia, myalgias, nausea, rash, and fatigue. Grade 3 adverse events in the first course included hypophosphatemia ($n = 10$), hyperglycemia ($n = 3$), hypoxia ($n = 2$), hypotension ($n = 1$), thrombocytopenia ($n = 1$), aspartate aminotransferase elevation ($n = 2$), and hyperbilirubinemia ($n = 1$). Dose-limiting toxicities, all of which resolved spontaneously, were consistent with those expected on the basis of prior studies of IL-2 or anti-GD₂ monoclonal antibodies administered alone. Based on the IL-2-related toxicities of hypoxia and hypotension, 7.5 mg/m²/d for 3 d was considered the maximum tolerated dose in this study (32). hu14.18-IL-2 caused immune activation as reflected in rebound lymphocytosis, increased numbers of circulating CD16+ and CD56+ cells, and enhanced *in vitro* ADCC by peripheral blood mononuclear cells.

The half-life in serum of hu14.18-IL-2 appears to be 3.5 h. Serum peak levels exceeding 5 µg/mL are seen at doses of 6 or 7.5 mg/m²/d. This concentration is more than 100-fold the hu14.18-IL-2 level required for *in vitro* ADCC. To date, then, hu14.18-IL-2 appears to be generally well tolerated at doses that induce striking *in vivo* immunologic effects. A phase II study will evaluate antitumor activity and immunologic effects of this immunocytokine in subjects with advanced melanoma.

3.1.5. CLINICAL DATA: NEUROBLASTOMA

A similarly structured phase I trial of hu14.18-IL-2 is currently under way through the Children's Oncology Group at the University of Wisconsin (33). As of November 1, 2002, 16 children with recurrent or refractory neuroblastoma received hu14.18-IL-2 as

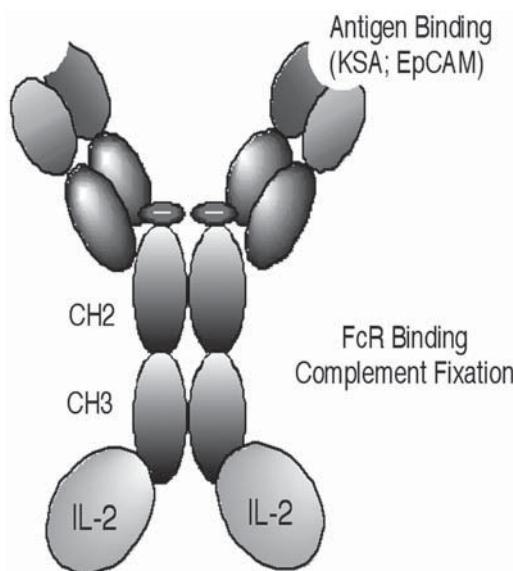


Fig. 3. Immunocytokine huKS-IL-2.

a 4-h intravenous infusion for three consecutive days. Children showing stable or improved disease are eligible for up to four courses of this 3-d treatment, repeated monthly. The immunocytokine has been generally well tolerated. Toxicities observed to date have been reversible and similar to those seen in prior pediatric trials of anti-GD₂ monoclonal antibodies and IL-2 (33). Separate groups of subjects have tolerated doses of 2, 4, 6, and 8 mg/m²/d by this regimen. The maximum tolerated dose has not yet been determined.

Upon completion of this phase I study, phase II testing in children with neuroblastoma is planned. This study will involve two cohorts of subjects. The first cohort will comprise subjects with clinically measurable recurrent or refractory disease. Because murine models show the most striking long-term effects of hu14.18-IL-2 in mice with small amounts of established tumor, rather than “bulky” disease, a second cohort of patients with minimal residual disease detectable only by sensitive immunocytochemistry of bone marrow samples or by sensitive ¹³¹I-M1BG-scanning will also be studied. The latter cohort will be evaluated for antitumor effect (based on marrow immunocytochemistry and M1BG-scanning) and on time to progression.

3.2. *huKS-IL-2 Immunocytokine*

Besides hu14.18-IL-2, one other immunocytokine—huKS-IL-2 (EMD273066; Fig. 3)—is currently being studied in patients. Immunocytokine huKS-IL-2 is composed of two molecules of IL-2 genetically fused to a humanized antibody directed against human adenocarcinoma-associated antigen (KSA, also known as EpCAM, or epithelial cell adhesion molecule), which is highly expressed on many epithelial cancers including prostate, colon, breast, and lung (34,35). Each immunocytokine molecule comprises two IL-2 molecules fused to each of the two heavy chains of the monoclonal antibody via a peptide linkage at the carboxyl terminus.

3.2.1. BIOLOGICAL ACTIVITY

Early studies of the biological activity of huKS-IL-2 demonstrate that the immunocytokine retains full antibody and cytokine functions. That huKS-IL-2 retains full antibody properties is illustrated by the finding that the immunocytokine and the anti-EpCAM antibody had identical K_d values (2.6 nM) and numbers of binding sites per cell (7.2×10^5) in a direct binding assay involving human prostate carcinoma cells (PC-3.MM2) (36). That huKS-IL-2 retains full cytokine properties was confirmed by the finding that the fusion of the anti-EpCAM antibody with IL-2 did not reduce IL-2 activity relative to that of commercially available IL-2 in proliferation assays using the mouse CTLL-2 lymphocyte line or human cell lines such as Kit-225 and TF-1beta (3,37).

3.2.2. ANTITUMOR ACTIVITY IN PRECLINICAL MODELS

The antitumor activity of huKS-IL-2 has been assessed both in xenograft models with SCID mice injected with human lymphokine-activated killer cells and in syngeneic models involving immunocompetent hosts. In syngeneic murine tumor models, murine tumors are transfected with the human KSA (EpCAM) gene to generate murine tumors recognized by the huKS monoclonal antibody, which is specific for the human EpCAM molecule. In syngeneic BALB/c mice, huKS-IL-2 at a dose of 15 µg/d for 7 d, but not comparable doses of the monoclonal antibody or IL-2 alone, eliminated established pulmonary and hepatic CT26/KSA colon carcinoma metastases (34). The amount of IL-2 required to stimulate antitumor responses was lower when IL-2 was administered as huKS-IL-2 than when IL-2 was given alone.

The ability of subtherapeutic doses of the chemotherapeutic agents paclitaxel and cyclophosphamide to augment the antitumor activity of huKS-IL-2 was recently assessed in several tumor models including CT26/KSA colon, 4T1/KSA mammary, and LLC/KSA Lewis lung carcinomas (35). One to 3 d after administration of a single dose of the chemotherapeutic agent, huKS-IL-2, was administered in five daily doses to mice with established subcutaneous tumors or liver or lung metastases. The results demonstrate that the chemotherapeutic agents added to huKS-IL-2 augmented antitumor activity relative to administration of huKS-IL-2 alone (Figs. 4 and 5; 35).

The mechanism of this enhancement of huKS-IL-2 effects when administered with chemotherapy is unknown. In an in vitro study, neither IL-2 nor huKS-IL-2 enhanced the cytotoxicity of paclitaxel, an observation that suggests that the antitumor effect was not attributed to increased paclitaxel mediated cytotoxicity in vivo (35). On the other hand, paclitaxel or cyclophosphamide treatment 24 h, but not 1 h, prior to administration of huKS-IL-2 markedly increased tissue uptake of huKS-IL-2 relative to that observed without pretreatment with chemotherapy. This finding suggests that these two chemotherapeutic agents may allow increased uptake of immunocytokine into the tumor microenvironment.

Besides demonstrating synergy with standard chemotherapeutic agents, tumor reactive immunocytokine administered in combination with an antiangiogenic integrin alpha_v antagonist was more effective than administration of the immunocytokine or the angiogenesis inhibitor alone in eradicating spontaneous tumor metastases in a syngeneic tumor model of neuroblastoma (38). The antitumor response was correlated with an approx 50% reduction in tumor vessel density and an increase in inflammatory cells in the tumor microenvironment.

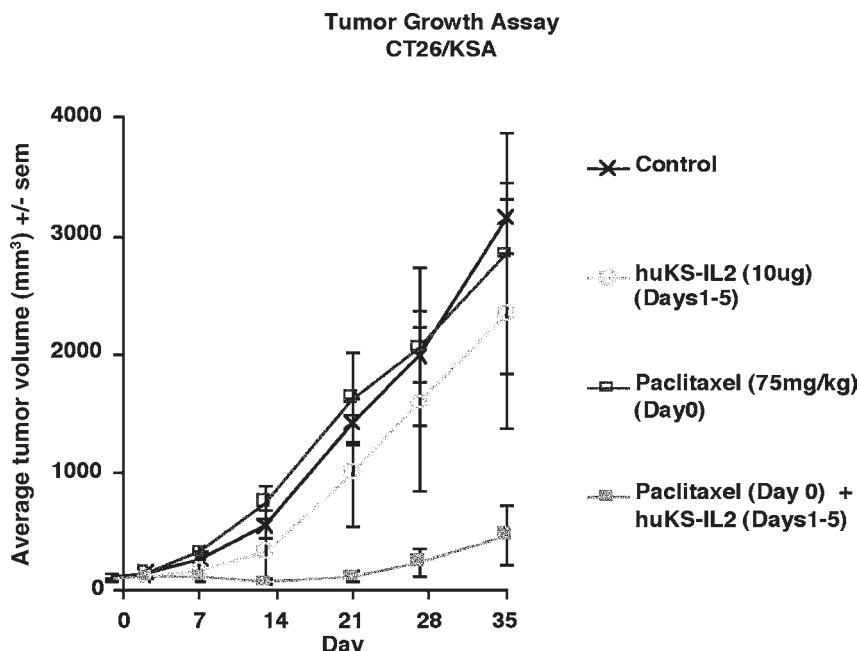


Fig. 4. Antitumor activity after combination therapy of huKS-IL-2 and subtherapeutic doses of paclitaxel in a CT26/KSA mouse colon carcinoma tumor growth assay. The figure depicts the average tumor volumes of seven mice per group from one representative experiment. On day 21, tumor volumes of the combination treatment group were significantly lower than those for the paclitaxel-alone group or the huKS-IL-2-alone group ($p < 0.05$). (Adapted with permission from ref. 35.)

3.2.3. IMMUNE MECHANISMS OF HUKS-IL-2

The mechanisms of the antitumor effects of huKS-IL-2 are not conclusively known, but research to date points to the involvement of both T-cell-dependent and T-cell-independent immune responses. The efficacy of huKS-IL-2 at eliminating pulmonary and hepatic colon carcinoma metastases of the originally derived CT26/KSA tumor was abolished in T-cell-deficient SCID mice and in mice depleted of CD8+ cells. These findings suggest that the antitumor activity of huKS-IL-2 in this model is CD8+ T-cell dependent (34). These findings are extended by the demonstration that administration of huKS-IL-2 to BALB/c mice with established pulmonary metastases of CT26/KSA colon carcinoma increased precursor cytotoxic T lymphocytes (CTLs), induced genes encoding Th1 cytokines, and generated tumor-specific CD8+ cells at magnitudes 10 to 14 times greater than those in control mice not administered huKS-IL-2 (39).

The demonstration of durable and transferable tumor immunity after administration of huKS-IL-2 is also consistent with a T-cell-dependent mechanism of antitumor activity. CD8+ T cells from splenocytes of tumor-bearing BALB/c mice treated with huKS-IL-2 were transferred to syngeneic SCID mice 6 wk prior to challenge with CT26/KSA tumor cells (39). Four days after the tumor-cell challenge, mice received boosters of huKS-IL-2, a mixture of huKS and recombinant human IL-2, or PBS (control). CD8+ T cells increased markedly in animals treated with huKS-IL-2 and hardly at all in control animals or animals treated with the antibody–cytokine mixture—a pattern of results

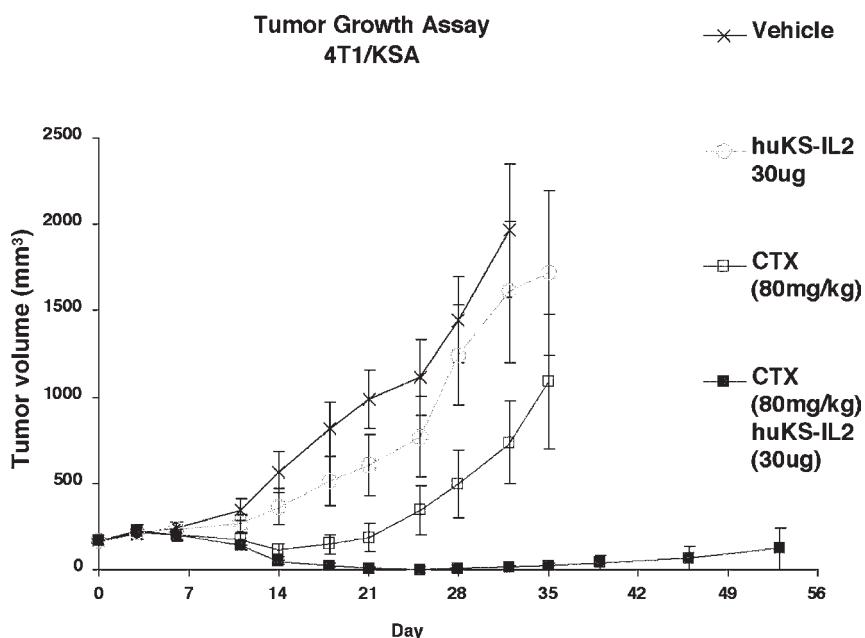


Fig. 5. Antitumor activity after combination therapy of huKS-IL-2 and subtherapeutic doses of cyclophosphamide in an intradermally injected 4T1/K5A mouse breast carcinoma tumor growth assay. The figure depicts the average tumor volumes of seven to eight mice per group from one representative experiment. On day 21, tumor volume of the combination treatment group was significantly lower than that for the huKS-IL-2-alone group ($p < 0.05$). (Adapted with permission from ref. 35.)

presumed to reflect presence of T memory cells that recognized the tumor antigen 6 wk after initial exposure. CD8+ cells from mice boosted with huKS-IL-2, but not with PBS or the antibody–cytokine mixture, were able to kill tumor cells in vitro. A similar experiment demonstrates that CD8+ T cells transferred from immune mice to syngeneic SCID mice could maintain durable memory in the absence of tumor antigen and naïve T cells (39). CD8+ cells from animals cured of CT26/KSA were “parked” for 6 wk in naïve SCID mice. They were then harvested and injected into a second set of naïve mice 48 h before an intravenous injection of CT26/KSA. Mice receiving the CD8+ cells derived from the immune animals showed a dramatic decrease in lung weight and lung metastases compared to animals getting control CD8+ cells. This antitumor memory response was further enhanced by subsequent administration of the KS-IL-2 immunocytokine. Prevention of establishment of pulmonary metastases was observed only in mice that had received CD8+ cells (putatively, memory cells) from mice that (a) had been successfully treated with huKS-IL-2 and (b) were later boosted with two noncurative doses of huKS-IL-2 (Table 3; 39). huKS-IL-2 boosters administered to mice that had not received CD8+ cells from successfully treated animals were not effective in preventing the establishment of pulmonary metastases. These data are consistent with the possibility that tumor cell challenge and an immunocytokine boost induced differentiation of resting CD8+ T memory cells into tumor-specific CD8+ T effector cells with effective antitumor activity.

Whereas these findings convincingly establish a T-cell-dependent mechanism of anti-tumor effects of huKS-IL-2 in this CT26/KSA model, other data suggest that T-cell-

Table 3
Horizontal Transmission of Tumor Immunity by Adoptive Transfer of CD8+ T Cells^a

<i>Therapy to donor</i>	<i>Transfer</i>	<i>Boost</i>	<i>Metastatic score^b</i>	<i>Lung weight, g</i>
None	None	None	3,3,3,3	0.99 ± 0.10
PBS	CD8+	None	3,3,3,3	0.85 ± 0.10
PBS	CD8+	huKS-IL-2	3,3,3,3	0.91 ± 0.12
huKS + IL-2	CD8+	huKS-IL-2	1,2,2,2	0.41 ± 0.04
huKS – IL-2	CD8+	ch14.18-IL-2	0,1,1,2	0.37 ± 0.07
huKS – IL-2	CD8+	None	0,1,1,1	0.32 ± 0.03
huKS – IL-2	CD8+	huKS-IL-2	0,0,0,0	0.21 ± 0.01

Adapted with permission from 39.

^aBALB/c mice cured of pulmonary CT26/KSA metastases after treatment with huKS-IL-2 2 wk after tumor cell inoculation (or control PBS-treated or huKS + IL-2-treated mice) served as donors for CD8+ T cells adoptively transferred to syngeneic SCID mice. CD8+ T cells purified from splenocytes were injected intravenously into BALB/c SCID/SCID mice and parked there for 6 wk. Then, 48 h before intravenous challenge with CT26/KSA tumor cells, naïve syngeneic SCID mice were reconstituted with CD8+ T cells via intravenous infusion on days -1, +1, and +3. On days 4 and 6 after tumor-cell challenge, seven groups of mice (*n* = 4) received either no boost or two intravenous injections of a noncurative dose (5 µg each) of huKS-IL-2. One group of mice received the same regimen but with a nontumor-specific fusion protein (ch14.18-IL-2). Mice were sacrificed 28 days after tumor cell challenge and evaluated for metastatic disease.

^b0 = no visible metastatic foci; 1 = ≤ 5% of lung surfaces covered with metastatic foci; 2 = between 5 and 50% of lung surface covered with metastatic foci; 3 = ≥ 50% of lung surface covered with metastatic foci.

independent immune responses can also mediate antitumor effects of huKS-IL-2 (31). A variant subclone of CT26/KSA, designated CT26Ep-21.6, has a reduced expression of class I MHC molecules and is more sensitive to ADCC facilitated by KS-IL-2 and mediated by NK cells *in vitro* (40). This *in vitro* killing is enhanced by anti-Ly49 monoclonal antibody, which prevents the MHC class I molecules on the tumor from activating potent inhibitory signals in the NK cells via their Ly49 receptors (40). *In vivo*, the potent effect of KS-IL-2 against lung metastases of CT26 Ep-21.6 is dramatically inhibited by *in vivo* depletion of natural killer cells, a finding that suggests a NK-cell-mediated component of this effect.

In other models, administration of huKS-IL-2 to SCID mice with pulmonary metastases induced by intravenous injection of human prostate carcinoma cells (PC-3.MM2) suppressed tumor growth and dissemination at a dose of 2 µg and eliminated tumor growth at doses of 4 µg and above (Table 4; 36). Moreover, survival times in mice in which human prostate carcinoma metastases had been suppressed with huKS-IL-2 were four times higher than those in mice administered a mixture of unfused anti-KSA antibody and recombinant human IL-2. huKS-IL-2 effectively eradicated metastases in granulocyte-depleted SCID-beige mice lacking functional T cells, B cells, and NK cells, a finding that led the authors to speculate that macrophages were most likely responsible for the antitumor effect of the immunocytokine in this model under these experimental conditions (36).

3.2.4. CLINICAL DATA

Immunocytokine huKS-IL-2 is currently being evaluated in clinical studies as an immunotherapy for cancers of epithelial origin. A phase I clinical study conducted

Table 4
Effect of huKS-IL-2 on Lung Metastases
in SCID Mice

<i>Treatment^a</i>	<i>Metastatic Score^b</i>
PBS (control)	2,2,2,3,3,3
huKS-IL-2 ^c	
32 µg	0,0,0,0,0,0
16 µg	0,0,0,0,0,0
8 µg	0,0,0,0,0,0
4 µg	0,0,0,0,0,0
2 µg	0,1,1,1,1,1

Adapted with permission from 36.

^aMice received intravenous injections of PC-3.MM2 cells. Immunocytokine treatment was initiated 24 h postinoculation and was given in seven daily intravenous doses.

^b0=no visible metastatic foci; 1 = ≤5% of lung surfaces covered with metastatic foci; 2 = between 5 and 50% of lung surface covered with metastatic foci; 3=≥50% of lung surface covered with metastatic foci

^c*p* < 0.01 vs PBS control.

primarily to evaluate the safety of huKS-IL-2 has been completed (41,42). In that study, which enrolled 22 patients with advanced hormone-refractory prostate cancer, huKS-IL-2 was administered in a dose-escalation protocol (0.35 mg/m² to 8.5 mg/m²) in a 4-h infusion daily for three consecutive days. It was generally well tolerated up to a dose of 4.3 mg/m²/d. The maximum tolerated dose was 6.4 mg/m²/d. huKS-IL-2-associated adverse events and toxicities, such as rigors, chills, anemia, and signs of vascular leak syndrome, occurred predominantly at doses exceeding 4.3 mg/m²/d and are consistent with the established side-effect profile of IL-2 and with the effects of antibodies administered alone (43,44). huKS-IL-2 demonstrated biologic activity as measured by increases in total lymphocyte counts, NK-cell counts, NK-cell activity, and ADCC in the peripheral circulation. The magnitude of treatment-related increases in these parameters ranged from 50 to 330% over pretreatment baselines, and the increases generally persisted for 3–4 wk. Lymphocyte counts demonstrated known IL-2 effects of lymphopenia during treatment followed by rebound lymphocytosis with each cycle of administration. These data showing broad-based immunologic activity of huKS-IL-2 in patients with prostate cancer are consistent with results of animal studies demonstrating that the antitumor activity of huKS-IL-2 can be effected via either T-cell-dependent or T-cell-independent mechanisms.

3.3. Non-IL-2-Based Immunocytokines

3.3.1. IL-12-BASED IMMUNOCYTOKINES

On the basis of the promising preclinical and clinical data with immunocytokines incorporating IL-2, attempts have been made to target other cytokines such as IL-12 to the tumor micro-environment via delivery as immunocytokines. Like IL-2, IL-12 potently

stimulates NK cells and CD8+ cytotoxic T lymphocytes, but IL-12 more potently stimulates interferon- γ and induces Th1 cell differentiation than does IL-2 (11). Several fusion proteins composed of antitumor antibodies and IL-12 have been tested for biological activity and antitumor efficacy in animal models (45). Although the biological activity of the IL-12-based immunocytokines studied to date has generally been suboptimal, antitumor efficacy has been demonstrated for established pulmonary metastases of CT26/KS colon carcinoma in SCID mice as well as for metastatic prostate carcinoma xenografts in SCID mice transplanted with human lymphocyte-activated killer cells (45). Recently, the cytokine combination IL-2/IL-12 was successfully fused with an antibody in an attempt to improve upon the cytokine activity of IL-2 or IL-12 alone (46). This bifunctional cytokine fusion protein shows potent antitumor activity in established tumor models.

3.3.2. GRANULOCYTE-MACROPHAGE-COLONY STIMULATING FACTOR-BASED IMMUNOCYTOKINES

An immunocytokine composed of GM-CSF and chimeric or humanized anti-GD₂ antibody 14.18 is also being studied in animal models (47,48). GM-CSF enhances granulocyte- and monocyte-mediated ADCC and has been used in combination with various antibodies in clinical trials of patients with colorectal carcinoma and neuroblastoma with positive results (49–51). The biological activity of ch14.18/GM-CSF immunocytokine was confirmed in a series of studies showing that the immunocytokine was as effective as equivalent amounts of its antibody and cytokine components in facilitating granulocyte-mediated killing of NMB7 neuroblastoma cells and enhancing ADCC by mononuclear cells from patients with neuroblastoma (47). In another series of studies, hu14.18/GM-CSF facilitated polymorphonuclear leukocyte-mediated ADCC against neuroblastoma cells (47).

3.3.3. OTHER IMMUNOCYTOKINES

The data from animal models described above provide an *in vivo* proof of principle. They show that delivery of cytokines by tumor-reactive antibody to the *in vivo* tumor microenvironment induces potent T-cell-mediated and NK-cell-mediated effects and *in vivo* antitumor destruction that exceed those obtained with treatment with monoclonal antibodies or cytokines alone or in combination. As such, similar constructs using other tumor-reactive monoclonal antibodies fused to IL-2, IL-12, or other cytokines are also expected to provide enhanced antitumor efficacy over treatment with monoclonal antibodies alone or combined with soluble cytokines (52–54).

4. CONCLUSIONS

The preclinical and early clinical data on immunocytokines for cancer therapy suggest that this new therapeutic modality has promise for improving outcomes for patients with cancer. As administration of immunocytokines poses no apparent risk of cross-resistance with conventional therapeutic modalities including chemotherapeutics, radiotherapy, and surgery, combination therapy appears to be feasible and should be explored. Preclinical data suggest that the greatest long-term tumor protection by immunocytokines is obtained in animals with minimal tumor burdens. Thus, regimens that include immunocytokines merit development and testing in experimental animals and patients achieving clinical remission via conventional treatments but likely to experience

recurrence. Combination therapy involving chemotherapeutic agents and immunocytokines, in particular, may be a fruitful approach given the preclinical work demonstrating synergy of immunocytokines and standard chemotherapies with respect to antitumor effects. Besides combination therapy involving immunocytokines and standard cancer treatments, combinations involving immunocytokines and other immunomodulatory approaches such as gene therapy may constitute a promising avenue for additional research and have begun to be assessed in preclinical models (55).

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CONTENTS

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1. INTRODUCTION

Adoptive transfer originally referred to the ability to confer protective immunity on a naïve host via infusion of T lymphocytes from an immune donor. This term now also encompasses a strategy of cancer therapy in which autologous T cells are acquired from a tumor-bearing host then activated and numerically expanded ex vivo prior to reinfusion. It has been nearly 50 yr since Mitchison's initial observation that adoptive transfer of "cellular elements" from immune hosts could accelerate rejection of tumor transplants in naïve recipients (1). Since then, considerable progress has been made in defining T lymphocytes as the central component of the antitumor response with the ability to directly kill tumor cells and orchestrate other host effector mechanisms. With the importance of T cells firmly established, tumor-reactive T-cell clones have been successfully used as probes to identify tumor-associated antigens that are currently being investigated as vaccine reagents.

The vast majority of current strategies of cancer immunotherapy involve treatment of established disease rather than prophylaxis; therefore, it is necessary to contend with the preexisting state of the immune system in the tumor-bearing host. There is considerable experimental and clinical evidence indicating both global and antigen-specific defects in

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the function of antigen-presenting cells (APCs) as well as T cells in cancer patients. These defects in immune function are not insurmountable, as evidenced by durable complete responses to immunotherapy (2). However, immunosuppression may be one factor that limits the effectiveness of active immunotherapy. It is also clear from animal studies that efficacy of T-cell immunotherapy is dose dependent. Therefore, it is anticipated that large numbers of effector cells will be required to treat more challenging clinical situations such as disseminated or advanced bulk disease. These considerations provide the rationale for adoptive immunotherapy, namely that tumor-reactive T cells can be optimally activated and expanded to large numbers ex vivo, free of suppressive factors. Meanwhile, the host can undergo chemotherapeutic or immunomodulatory therapy without detrimental effects on the extracorporealized effector T cells.

Adoptive transfer is also a very powerful tool in animal models because it permits ex vivo selection, manipulation, and tagging of independent T-cell subsets in order to study their function following return to the tumor-bearing host. Currently, the level of understanding of mechanisms involved in immunotherapy of spontaneously arising human cancer still lags behind that of experimental animal models and it is not yet known whether there will be critical differences. Moreover, the response rates obtained in cancer immunotherapy trials are not nearly high enough to simply allow empirical refinement. These considerations provide a second rationale for the development and clinical investigation of adoptive immunotherapy, because it will permit a more systematic analysis of the components, dose, and schedule of T cells that will be required to achieve complete tumor regression in human cancer patients. Such information will in turn aid the design of active immunotherapy strategies.

Animal models of adoptive transfer of tumor-sensitized T cells have elucidated many important parameters that have influenced the design of clinical trials. Some of the relevant topics that will be discussed are the source of T cells, methods for enrichment of tumor-sensitized T cells, and conditions for ex vivo activation and propagation. A powerful tool has been the use of donors or recipients that are genetically defective for various immune functions and these studies will be discussed. Methods of T-cell preparation that preserve trafficking and effector function are important, as are the influence of cytokines and costimulatory molecules on efficacy. Despite the powerful advantages of preclinical animal models such as the ability to use appropriate experimental controls and to dictate tumor load and timing of therapy, they nevertheless utilize rapidly growing tumor lines and transfer between syngeneic animals. This falls short of completely recapitulating the clinical situation where the host immune system has already been shaped by interactions with the tumor. Thus, the incomplete picture provided by animal models requires supplementation with data from clinical trials.

The published clinical experience with T-cell immunotherapy still consists mainly of descriptive rather than functional studies and the number of such studies is relatively small. These trials have used several sources of T cells such as peripheral blood lymphocytes (PBLs), tumor-infiltrating lymphocytes (TILs), and vaccine-draining lymph node (VDLN) cells as well as a variety of methods of ex vivo activation. Most of these are phase I clinical studies that have focused on the feasibility and toxicity of the treatment, although there have been several randomized controlled trials. Nevertheless, progressive improvements in tumor vaccines and T-cell culture methods have brought adoptive immunotherapy closer to practical clinical use. More importantly, clinical responses attributable

to transferred tumor-reactive T cells have been clearly documented, thereby establishing T-cell adoptive immunotherapy as a valid approach to cancer therapy.

2. EARLY STUDIES OF ADOPTIVE IMMUNOTHERAPY OF TUMORS

Two of the fundamental concepts that underlie tumor immunotherapy, namely that cellular elements could confer immunity upon naïve recipients and that unique tumor antigens were the relevant targets, were established empirically long before T lymphocytes were defined or the nature of antigen recognition was understood at a molecular level. Billingham observed that immunity to skin grafts could be transferred to naïve recipients by lymph node (LN) cells and introduced the term “adoptive immunity” to describe this phenomenon (3). This concept was soon extended to transplantable tumors by Mitchison (1). In his pioneering study, Mitchison documented the efficacy of TDNL cells rather than distant LNs or peripheral blood, the requirement for viable lymphoid cells, the dependence on a sufficient cell dose for protection, and the development of a memory response. Klein demonstrated that primary hosts rendered surgically free of carcinogen-induced tumors and subsequently immunized with irradiated tumor fragments could reject live autochthonous tumor. This study conclusively distinguished tumor rejection antigens from any heterogeneous antigens that might remain among members of inbred strains (4). It is important to retain sight of potential distinctions between the immune response against major or minor histocompatibility antigens following stem cell transplant vs rejection antigens on autochthonous tumors when comparing strategies for immunotherapy.

Studies conducted by Prehn and Main used live tumor to stimulate a response followed by challenge of mice surgically rendered tumor-free (5). This study demonstrated the existence of tumor-specific rejection antigens on carcinogen-induced tumors that were stable over many transplant generations and were, to a large extent, not shared between tumors of similar histology. An additional finding with potential relevance to clinical applications of cancer immunotherapy was that several spontaneously arising fibrosarcomas were very poor at inducing protective immunity in comparison to the carcinogen-induced tumors. The relative immunogenicity of spontaneous vs carcinogen-induced tumors and whether there are qualitative differences in the nature of the immune response in these two situations provided fuel for debate (6). Recently, the creation of transgenic animal models with genetically induced tumors of targeted tissues, coupled with the ability to express defined “model tumor antigens” within these tumors, has shifted the ground but has not resolved the debate about what constitutes a satisfactory animal model of spontaneously arising human cancer.

There were several aspects of the early animal models of adoptive immunotherapy that precluded their direct application to human cancer patients. Firstly, the donors of the lymphocytes were tumor immune rather than tumor bearing. Secondly, cells were pooled from several donors to treat each recipient. Moreover, the experimental design was typically protection from challenge rather than treatment of established tumors, although the latter was successfully demonstrated (7,8). The results from animal studies clearly demonstrated a need for donor lymphocytes that were primed by prior tumor exposure and specificity for unique tumor antigens. Nevertheless, a number of early clinical studies of adoptive immunotherapy used lymphoid and myeloid cells from major histocompatibility complex (MHC)-matched donors but without attempts at prior sensitization to

tumor antigens. Studies where prior sensitization with tumor was employed used cross-immunization between pairs of cancer patients or tumor immunization of normal donors followed by adoptive transfer of peripheral blood leukocyte or spleen cells but without human leukocyte antigen (HLA) matching. These early clinical studies of adoptive immunotherapy failed to demonstrate substantial therapeutic benefit and have been comprehensively reviewed (9).

3. CRITICAL DEVELOPMENTS FOR ADOPTIVE IMMUNOTHERAPY USING AUTOLOGOUS T CELLS

Clinical applications of adoptive immunotherapy will likely be most effective and least toxic if they use an autologous source of tumor-reactive T cells. T cells recognize tumor antigens in the form of peptide fragments of proteins presented by MHC molecules. Thus, each tumor antigen that is targeted must be matched for the relevant MHC molecule. Additionally, humans have a large number of alleles in the HLA gene complex and a mismatch of donor and recipient at either class I or class II loci leads to rapid rejection of transferred effector cells or alternatively risk of graft vs host disease.

The source of autologous T cells is important because successful adoptive immunotherapy is dependent on the dose of tumor-reactive T cells. The effector cell number is the product of the precursor frequency in the sample multiplied by the total number of cells generated. Therefore, identification of sources of T cells that are already considerably enriched for tumor-reactive precursors is critical. Alternatively, a method to purify tumor-reactive T cells from a complex mixture *ex vivo* prior to numerical amplification is desirable. Likewise development of methods to numerically expand T cells to large numbers while retaining their ability to function *in vivo* following adoptive transfer is important.

The availability of recombinant interleukin-2 (IL-2) and improved cell culture techniques made clinical application of adoptive immunotherapy a reasonable prospect. The first experiments to investigate the use of IL-2-activated T lymphocytes tested cells from immune animals. These studies were performed prior to the availability of recombinant human IL-2 and used supernatant from concanavalin A-stimulated murine T cells as a source of IL-2. Cheever and colleagues recovered 200% of the input cells after 19 d in culture and showed specificity in cytolytic activity and in prolongation of survival using FBL-3 and EL-4 models of chemoimmunotherapy (10). In a similar strategy, mice were cured of FBL-3 tumors following adoptive transfer of cells expanded greater than 3000-fold (11).

A critical advance for the field of adoptive immunotherapy was the demonstration that lymphocytes could be derived from a host with a progressively growing tumor rather than tumor-free immune animals. This experimental scenario is much more analogous to clinical situation. These experiments are noteworthy because prior attempts to treat tumors by adoptive transfer of lymphocytes from hosts bearing progressive tumors were unsuccessful. The first applications used lymphokine-activated killer (LAK) cells. Although LAKs turned out not to be classical T cells or to exhibit MHC-restricted lysis of targets, the ability to obtain them from spleen or peripheral blood of tumor-bearing hosts generated interest (12). Derivation of effector T cells from TILs, or from draining LNs and spleen was the next major development (13,14). In these experimental models spleen and tumor-draining LNs (TDLNs) were free of tumor, therefore, the function of the T cells

in secondary hosts could be compared immediately after acquisition vs following ex vivo propagation.

An important feature of T cells derived from tumor-bearing mice is that they only develop potent effector function following ex vivo activation, whereas freshly acquired cells have undetectable efficacy (14,15). The initial experimental strategy, termed in vitro sensitization (IVS), used tumor cells as a source of antigen during culture activation. Spleen cells or TDNL cells were culture activated with irradiated tumor cells and IL-2 (1000 U/mL) for a 9- to 14-d period and underwent approx 100-fold numerical expansion. The presence of irradiated tumor cells as a source of antigen during in vitro activation was essential, as IL-2 treatment alone did not generate effector function. The biochemical basis for the acquisition of effector function by ex vivo activation was explored through comparison of signal transduction molecules in freshly acquired vs culture-activated TDNL cells. Fresh LN cells showed a decreased amount of *lck* and *fyn* as well as diminished tyrosine phosphorylation of substrates upon T-cell receptor (TCR) stimulation but these abnormalities were corrected after in vitro activation (16). Signaling defects have also been extensively documented in PBLs of patients with advanced cancer and in TILs (17–20). The dramatic improvement in effector function mediated by ex vivo activation combined with the numerical amplification of precursors remains to this day the fundamental principle for adoptive immunotherapy in situations where active immunotherapy is subtherapeutic.

Another innovation that helped clear a hurdle to adoptive immunotherapy was the demonstration that stimulation of TDNL cells or immune lymphocytes with anti-CD3 mAb and IL-2 was effective for ex vivo activation (21,22). This was important from a practical standpoint because antigen-independent stimulation freed investigators from the constraint of needing a large quantity of autologous tumor cells as a reagent for T-cell activation. Additionally, IVS with tumor cells resulted in the preferential activation of CD8+ T cells whereas anti-CD3 stimulation promoted the expansion of both CD4+ and CD8+ subsets. Several other methods of antigen-independent T-cell activation have been developed, including bacterial superantigens or bryostatin and ionomycin (23,24). In all of these systems, there is a requirement for the T cells to be previously sensitized to specific tumor antigens in vivo prior to antigen-independent ex vivo activation. T cells obtained from normal mice and activated with anti-CD3 and high-dose IL-2 demonstrated extensive T-cell proliferation in vitro but displayed minimal in vivo efficacy against established tumors and without clear documentation of specificity or generation of a memory response (25,26).

4. SALIENT FEATURES OF ADOPTIVE IMMUNOTHERAPY: PRECLINICAL LESSONS

4.1. *Enriched Sources of Tumor-Reactive T Cells*

T cells reactive to specific tumor antigens are likely to have a low precursor frequency. This indicates that in vivo or in vitro methods to enrich them are required. The process of naïve T-cell sensitization in secondary lymphoid tissue and mature effector trafficking into peripheral tissue suggest two logical in vivo sources that would be highly enriched for tumor-reactive T lymphocytes, namely VDLNs and TILs. In formative experiments using TILs, the initial ex vivo activation stage contained viable tumor cells, stromal cells,

and lymphocytes. In order to provide the T cells with a proliferative advantage over tumor and to hasten cytotoxicity, cells were maintained in the presence of high concentration of IL-2 (1000 U/mL). The tumor cells and passenger APCs provide a source of antigen during the early phase of ex vivo proliferation. As the TIL cultures mature, the source of antigen is extinguished and proliferation is maintained through persistent signaling through the IL-2R, which at high IL-2 concentrations needn't depend on the high-affinity form of the receptor. In the initial reports, TILs were isolated from murine tumors of diverse histologic types, raising the possibility of a readily available source for clinical use (27). This enthusiasm was tempered somewhat as several features of TILs emerged in further preclinical testing. TILs generated in high-dose IL-2 displayed both tumor-specific and nonspecific effects in vivo. In addition, TILs were difficult to generate from nonimmunogenic tumors and had limited efficacy against tumor in several anatomic sites. Some of these limitations were successfully overcome by Yang et al. through modifications including use of irradiated tumor during culture activation and maintenance of TILs in low concentrations of IL-2 (10 U/mL) (28). Although these conditions permitted TIL therapy of nonimmunogenic tumors, they also limited the total number of cells that could be routinely obtained from a single tumor-bearing host.

VDLNs are another highly enriched source of sensitized T cells. The trafficking pattern of naïve T cells through secondary lymphoid tissue rather than peripheral tissue combined with the predominant acquisition of antigens by dendritic cells (DCs) in tissues followed by migration through lymphatics to draining LNs favor this source. An extensive preclinical experience has been obtained using draining LNs for tumors of various histologic types including nonimmunogenic tumors. In all tested models, TDNLs become hyperplastic in several days and remain enlarged even though metastatic tumor cells are not normally detectable in the LN indicating a local immune response. There is a distinct kinetic pattern to the development of effector and suppressor immune responses. Tumor-reactive T cells can be identified as early as 6 d after tumor inoculation with the peak of activity at 9–12 d and waning after 14 d (21).

4.2. In Vitro Separation of Effector Cells

As might be anticipated for an antigen specific immune response, the tumor-reactive T cells are a minor fraction that bears a phenotype indicating recent activation, low expression of L-selectin (CD62L) (29). When freshly acquired TDNL cells were segregated based on L-selectin expression, the L-selectin^{low} subset comprising 5–15% of the T cells contained all the antitumor activity whereas the reciprocal L-selectin^{high} cells were completely ineffective (30). Similar findings were observed when T cells were segregated based on differential expression of P-selectin ligands (31). The L-selectin^{low} cells were 30-fold more effective on a per-cell basis than the original unfractionated population. The augmentation in efficacy was a result of elimination of irrelevant cells as well as suppressor cells. Recent studies have characterized populations of CD25+ regulatory T cells and their removal in vivo during tumor antigen sensitization increased the magnitude of effector T cells (32,33). The presence of established visceral tumor also suppressed the sensitization of T cells in LNs although the mechanisms involved have not been elucidated (34). Another class of suppressor T cells develops in draining LNs with a delayed kinetics relative to preeffector T cells. These suppressor T cells inhibit the function of effector T cells both in vitro and in vivo and can traffic to tumors to inhibit

the effector phase of the antitumor response (106). The differences in kinetics between maturation of effector and suppressor cells recommends a strategy of using LNs that are uninvolved with tumor as the source and obtaining draining LNs 6–12 d following antigen inoculation. Another promising strategy is to isolate T cells based on functional properties such as ability to secrete interferon (IFN)- γ upon ex vivo stimulation (35).

4.3. Trafficking of Transferred T Cells

If adoptive immunotherapy is envisioned for disseminated metastatic disease, it is essential that the activated T cells retain the capacity to traffic to all anatomic sites following adoptive transfer. We have confirmed the efficacy of adoptive immunotherapy with activated LN cells against tumor in all tested anatomic locations including lung, liver, subcutaneous tissue, and brain (36–38). Tagged T cells avidly infiltrate tumors in all anatomic sites although the initial infiltration of tumors by activated T cells is not antigen specific (39). It is essential that T cells retain the ability to traffic to tumors because if they are pharmacologically prevented from undergoing diapedesis they lose efficacy when systemically transferred even though they are still functional when coinoculated in a Winn assay (40). Treatment with high-dose IL-2 has been used in conjunction with T-cell adoptive immunotherapy in most applications with the rationale that it preserves the viability and proliferative capacity of effector T cells. IL-2 is not absolutely required for T-cell function, and in models such as treatment of pulmonary metastases where it augments efficacy similar results can be obtained through use of a fourfold escalation in T-cell dose. Furthermore, in certain models, such as treatment of intracranial or subcutaneous tumors, IL-2 dramatically inhibits therapeutic efficacy (38,41). The principal reason is that IL-2 inhibits trafficking of T cells to these sites (42). These preclinical observations led to modification of clinical trials of adoptive immunotherapy of brain tumors to eliminate conjunctional IL-2. Apparently, the response of TILs to concomitant IL-2 is somewhat different than VDLN cells because IL-2 does not completely prevent trafficking of TILs to visceral and subcutaneous tumors (43–45). In contrast to IL-2, treatment of recipients of T cells with MAb directed at costimulatory molecules such as OX-40R stimulates vigorous proliferation of effector cells that have migrated to tumors and augments therapeutic efficacy (42).

4.4. Multiple Effector Mechanisms Contribute to Tumor Regression

There are several effector mechanisms that contribute to regression of established tumors by adoptive transfer of T cells. In the L-selectin^{low} subset of T cells derived from VDLNs either CD4+ or CD8+ T cells can independently mediate tumor regression (46,47). Interestingly, the relative efficacy of CD4+ or CD8+ cells is dependent on the anatomic site of tumor with intracranial and subcutaneous sites being more CD4 dependent and pulmonary tumors more CD8 dependent. In the tumor models employed for these studies the tumor cells express only MHC class I molecules whereas MHC class II is not expressed nor is it induced by IFN- γ . This indicates that indirect presentation of tumor antigens by host APCs to transferred CD4+ T cells is sufficient to initiate tumor regression (48). This concept was confirmed and extended to CD8+ cells using adoptive transfer of effector T cells into tumor-bearing bone marrow chimeras under conditions that restricted antigen presentation to host tumor-infiltrating APCs and eliminated direct T-cell-tumor-cell interactions (49). In addition to sufficiency of CD4 and CD8 subsets,

there also appears to be participation of multiple effector molecules. Use of knock-out hosts that are genetically deficient in various effector molecules demonstrated that perforin-dependent lysis of target cells and IFN- γ -mediated mechanisms are each important but are not absolutely required (50–52).

Taken together, these preclinical observations indicate that T cells sensitized to specific tumor antigens can be isolated from tumor-bearing hosts and are enriched in TILs and VDLNs. Moreover, the subset of tumor-sensitized T cells can be substantially purified based on differential expression of activation markers on the cell surface. Ex vivo stimulation of sensitized T cells does not require reexposure to tumor antigen but can be suitably performed with anti-CD3 mAb and this process dramatically improves the functional capacity of the cells as well as increasing their number. Proper activation conditions preserve the ability of the activated T cells to traffic to tumor in all anatomic locations. These conditions stimulate both CD4+ and CD8+ cells that employ multiple redundant effector mechanisms.

5. CLINICAL EXPERIENCE WITH T-CELL ADOPTIVE IMMUNOTHERAPY OF CANCER

5.1. *Lymphokine-Activated Killer (LAK) Cells*

LAK cells are derived from peripheral blood lymphocytes cultured in the presence of high concentrations of IL-2 for 3–4 d. They demonstrate preferential lysis of tumor cells but not normal cells. Promising data in animal tumor models and preclinical in vitro testing raised the prospect of a readily available source of autologous cells with broad reactivity (12,53,54). Analysis of the relevant precursors of LAK cells indicated a natural killer (NK) origin and the treatment effect was also independent of host T cells (55–57). The first clinical studies of LAK cells were performed at the National Cancer Institute (NCI) surgery branch and used total cell doses ranging from 1.8 to 18.4×10^{10} cells combined with bolus high-dose IL-2 (720,000 U/kg). This study showed the greatest efficacy for patients with malignant melanoma or renal cell carcinoma with a 44% overall response rate (58). Based on this promising initial study, several phase II clinical trials of LAK cells were performed in the late 1980s. Two trials for melanoma patients, with 33 and 32 patients, respectively, used either bolus IL-2 or continuous-infusion IL-2 with response rates of 19% and 3% (59,60). The toxicity of these regimens was transient but substantial, routinely requiring intensive care and frequently ventilator support, limiting its use to patients with a good performance status and adequate organ function (61). A comparison of results from two European studies one using IL-2 alone and the other IL-2 with LAK cells suggested that IL-2 effects substantially accounted for the treatment efficacy (62). A prospective randomized trial performed at the NCI surgery branch likewise did not reveal a statistically significant difference between IL-2 alone or IL-2 with LAK cells (63). The failure to consistently observe therapeutic gains above those achieved with IL-2 alone coupled with the logistical burden and expense of generating large numbers of cells ex vivo led investigators to pursue enriched sources of tumor-reactive T lymphocytes rather than LAK cells for systemic therapy.

One of the difficulties noted with LAK cells is that they do not traffic well to peripheral sites of tumor. This has led several investigators to refine LAK therapy through delivery into anatomically localized tumor resection cavities, where poor trafficking is not a

limiting factor. Instillation of LAK cells into the resection cavity of patients with malignant brain tumors has led to several instances of tumor regression and prolongation of survival beyond historical controls (64–68). In a related strategy, Kruse has explored the use of allogeneic cytolytic T cells (CTLs) for local instillation into the resection cavity of malignant brain tumors (69,70). The expression of MHC class I molecules on malignant brain tumor cells but lack of expression on normal brain tissue is hypothesized to direct the allogeneic response toward the malignant cells. Cloned NK cells with high levels of perforin- and granzyme-mediated cytolysis are also in early stages of clinical investigation (71). In general, localized adoptive immunotherapy with LAK cells or CTLs is well tolerated and has resulted in several long-term responses, although efficacy has not yet been rigorously confirmed in a prospective randomized clinical trial.

5.2. Peripheral Blood Lymphocytes (PBLs)

Antigen-experienced T cells with a memory phenotype preferentially circulate in the peripheral blood. One of the theoretical advantages of using PBLs is that they are readily accessible and can be safely harvested in large quantities through standard leukapheresis procedures. Several studies tested the clinical efficacy of PBL activated with anti-CD3 and IL-2 termed autolymphocyte therapy (ALT) in patients with metastatic RCC (72,73) or metastatic melanoma (74). Simultaneous CD3 and CD28 stimulation by mAb coupled to magnetic beads in the presence of IL-2 (100–400 IU/mL) has also been tested on PBL from cancer patients resulting in 10- to 15-fold numerical expansion after 14 d of culture (75). The PBL stimulated with anti-CD3 alone had equivalent to slightly improved effector function in cytolytic and cytokine release assays compared with combined anti-CD3/anti-CD28 stimulation. Other studies also raise the issue of whether costimulation through CD28 is required to generate superior function in memory T cells (76). Therapy with PBL costimulated with anti-CD3/anti-CD28-coated beads for 4 d followed by culture in IL-2 (100 IU/mL) for an additional 10 d has been evaluated in a phase I trial. Ten patients received doses of activated T cells up to a maximum of 10^{10} per dose delivered twice a week for 3 wk with a total cell dose range of $1.7\text{--}4.2 \times 10^{10}$ cells. The treatment was well tolerated and subjects developed increased levels of cytokines in serum following therapy (77).

An interesting extension of PBL therapy that has been considered by several investigators involves increasing the precursor frequency of antigen-specific T cells in peripheral blood by vaccination prior to leukapheresis and ex vivo activation. Indeed, analysis of precursor frequency has shown an increase following peptide vaccination (78–80). It is possible that application of sophisticated methods to identify and purify antigen-specific T cells from a complex mixture in peripheral blood will bring this strategy into active clinical testing in the near future (35,81).

5.3. Tumor-Infiltrating Lymphocytes

T lymphocytes obtained from tumor deposits are presumably highly enriched for tumor-reactive effector memory cells that preferentially migrated into tumors and have been retained there. Enthusiasm for clinical use of TILs was engendered by murine tumor models that demonstrated tumor-specific cytolytic activity and cytokine release in vitro and regression of established tumors following adoptive transfer of relatively low cell numbers when compared with LAK cells (13,27,82). The most extensive clinical testing

of TIL therapy has been in patients with melanoma or renal cell carcinoma where unequivocal durable responses have been documented in patients with progressive bulky metastatic disease that was previously unresponsive to IL-2 alone.

One consideration for the clinical application of TIL therapy is that it requires considerable expertise and effort to successfully obtain therapeutic doses of TIL. Even the most experienced investigators are not uniformly successful. In a large series from the surgery branch at NCI describing the preparation of TIL from melanoma, renal, breast, or colon carcinoma, therapeutic numbers of greater than 10^{11} cells were successfully grown from 45% of the samples (83). In the initial methodology, TILs were derived from tumor biopsies that were enzymatically disaggregated and cultured in the presence of high concentrations of IL-2 (7200 IU/mL) and 10% autologous LAK cell culture supernatant. Among a variety of tumors, melanoma yielded the highest percentage of samples (37%) where preferential reactivity to autologous tumor was documented. In contrast, only 2 out of 49 samples derived from nonmelanoma histology resulted in TILs with the feature of preferential lysis of autologous tumor. Perhaps intrinsic properties of melanoma predispose this tumor to a high level of lymphocyte infiltration as indicated by greater than 50% of the viable cells from melanoma samples being lymphocytes. Interestingly, tumor reactivity was observed in 50% of TIL samples derived from subcutaneous melanoma compared with 18% of TIL derived from tumor-involved LNs. It is possible that abundant nonspecific passenger lymphocytes in LNs overgrew tumor-reactive T cells under the high IL-2 concentrations initially employed in TIL propagation favoring subcutaneous tumor deposits.

The overall response rate for patients with metastatic melanoma treated with TILs and conjunctional high-dose bolus IL-2 was 34% (84). This included a 6% complete response rate identical to that observed with high-dose bolus IL-2 alone. In keeping with the in vitro data, the clinical efficacy of TILs derived from subcutaneous melanoma was also higher (49%) than for TILs from tumor-involved LNs (17%). Additional variables that were associated with clinical responses included: shorter time in culture, more rapid doubling time, specific production of granulocyte-macrophage colony-stimulating factor (GM-CSF), and cytolysis to autologous tumor (85).

Several melanoma antigens recognized by TIL cultures have been defined at a molecular level. There was a correlation between reactivity with gp100 and clinical response (86). This led to strategies to specifically expand gp100 reactive clones from PBLs of patients vaccinated with modified gp100 peptides for treatment (87). Although purified gp100-reactive T-cell clones were administered at doses greater than 10^{10} cells in conjunction with IL-2, they displayed an insignificant level of therapeutic activity. The reason why a large dose of antigen-specific T cells did not function as well as TILs has not yet been defined. However, potential explanations could include progression of antigen-loss variant tumor cells or differences in trafficking. These parameters will hopefully be determined in future investigations.

Recently, adoptive immunotherapy of metastatic melanoma with TILs has been performed after conditioning the recipients with nonmyeloablative chemotherapy consisting of cyclophosphamide and fludarabine (88). This strategy led to dramatic in vivo clonal expansion of tumor-reactive T cells during concomitant IL-2 treatment, with several patients developing significant lymphocytosis. Unlike the previous clinical experience with TIL therapy, the nonmyeloablative preoperative regimen facilitated sus-

tained high levels of transferred T cells with the capacity to traffic to tumor and retention of reactivity to specific melanoma antigens. Partial response was observed in 6 of 13 patients with an additional 4 patients exhibiting mixed response. In addition, four of the responding patients developed vitiligo as has been previously observed in some melanoma patients responding to immunotherapy. The ability to develop dominant T-cell clones with sustained in vivo function in patients with metastatic melanoma represents a considerable therapeutic advance. The applicability of this approach to other tumors and the regulation of autoimmune effects will likely be areas of future exploration.

Renal cell carcinoma (RCC) is another reliable source of large numbers of TILs. Typically, after 3–5 wk in culture, the initial 10^8 – 10^9 lymphocytes obtained expand approx 100-fold to yield 10^{10} – 10^{11} TILs. Several clinical trials have been performed using variable culture conditions and different doses and schedules of concomitant IL-2. In one trial there were no responses among 18 patients indicating lack of sufficient effector cells in the initial sample or ineffective preservation of therapeutic activity during ex vivo propagation (89). Analysis of lymphocytes from within RCC samples showed that they do indeed have significant defects in signal transduction indicating one potential functional limitation (17,90,91).

A study of TIL therapy conducted at UCLA treated 55 patients using two strategies. The first used CD8 capture flasks for 23 patients with the rationale to purify effector CTL. The second approach used in vivo priming of TILs by treating patients with cytokines prior to nephrectomy (92). In this study, patients received IL-2 and IFN- α concomitantly with TIL. Overall, there was a 35% response rate with 9% complete response (CR) and a trend toward a higher response rate in the group treated with CD8+ TILs (43.5%). However, the differences in response rate did not reach statistical significance nor was there a difference in the duration of response between the two groups. Based on these promising results, a phase III multicenter double-blind randomized trial was conducted comparing continuous infusion IL-2 at a daily dose of 5×10^6 IU/m² with or without TILs (dose range 5×10^7 to 3×10^{10}). Reflecting some of the operational difficulties involved, there was a failure to generate a sufficient number of TILs in 41% of the patients and only 48% of the patients randomized to receive TILs actually received the therapy. The response rate in the TIL + IL-2 group was 9.9% compared with 11.4% in the IL-2 group; consequently the trial was discontinued because of failure to observe a difference in therapeutic effect (93). It is not apparent from the published data why the response rate was less than the preceding clinical trial. However, operational challenges involving the transport of tumor samples and the final TIL cell product from central processing facilities to treatment sites may have contributed to the high failure rate to generate a sufficient number of TILs. This study also reinforces the potential caveat that any processing or shipment of the T-cell product must retain the capacity of the cells to function in vivo. Development of in vitro correlates of in vivo efficacy may facilitate studies to optimize the preservation of T-cell function during preparation and transport.

TIL therapy has not yet found broad application for other common types of human cancer. It has been difficult to reliably generate suitable numbers of TILs from breast and colon carcinoma (94–97). The small sizes of typical biopsy specimens for prostate carcinoma and low number of lymphocytes have also hampered efforts to develop TILs for this disease. However, androgen withdrawal has been shown to increase infiltration of T cells with oligoclonal patterns of TCR expression, raising the hope of developing this

source if methods of ex vivo expansion can be optimized (98). TILs have also been derived from glioma specimens and delivered via local infusion into the resection cavity with several long-term responses (99). It will be of great interest to determine whether local administration of TILs is more effective than systemic administration for anatomically localized tumors that are refractory to current treatments, such as malignant glioma.

5.4. Vaccine-Draining LN T Cells

LNs draining a site of vaccine inoculation are an enriched source of T lymphocytes that have been sensitized to tumor antigens. Preclinical kinetic studies have clearly demonstrated that the optimal number of preeffector T cells is present between 6 and 14 d with subsequent waning of activity. These findings recommend a strategy of placing a tumor vaccine in a region that is uninvolved with tumor so that newly sensitized T cells can be obtained by removal of the vaccine draining LNs at the peak of the reaction.

In an initial study of VDLN cells, subjects with metastatic melanoma or RCC were treated. Irradiated autologous tumor cells admixed with bacille Calmette-Guérin (BCG) was used as a vaccine and 7 d later draining LNs were removed. The T cells were stimulated with immobilized anti-CD3 (OKT3) for 2 d followed by culture in IL-2 to reach a mean treatment dose of 8.4×10^{10} cells. These activation conditions generated a mixture of CD4 and CD8 that demonstrated specific release of GM-CSF and IFN- γ against autologous tumor targets. Eleven melanoma patients and 12 RCC patients were treated with T-cell infusion and concomitant IL-2. One patient with melanoma experienced a partial response (PR); two patients with RCC had a CR and two had a PR. Immune monitoring studies indicated a trend correlating development of a positive delayed-type hypersensitivity (DTH) response to autologous tumor with clinical response (100).

The feasibility of adoptive immunotherapy using VDLN cells was also tested in subjects with malignant gliomas. Two sequential trials were performed based on preclinical studies demonstrating that adoptive transfer of ex vivo-activated LN T cells could mediate regression of intracranial tumors including glioma (36,101). In a major departure from previous clinical studies of T-cell adoptive transfer, T cells alone were used for therapy without concomitant IL-2. This modification was based on findings that systemic IL-2 actually diminished the trafficking of adoptively transferred T cells into brain tumors and inhibited their therapeutic efficacy (38). In the first study, nine patients with recurrent glioblastoma multiforme (GBM) and one patient with anaplastic astrocytoma were vaccinated with irradiated autologous tumor cells mixed with GM-CSF (Sargamostim) as an adjuvant. Treatment with adjuvant GM-CSF was tolerated without toxicity and promoted local inflammation at the vaccine site but without skin ulceration that had occasionally been observed previously when BCG was used as an adjuvant. Draining LNs were removed 7 d later and stimulated with the bacterial superantigen *Staphylococcus aureus* enterotoxin A (SEA) and IL-2. The combination of SEA and a low concentration of IL-2 stimulated vigorous proliferation of both CD4+ and CD8+T cells. The cell dose ranged from 9×10^8 to 1.5×10^{11} cells and the treatment was tolerated without significant toxicity, leading to partial regression in two subjects (102).

A second cohort of patients with newly diagnosed gliomas was treated to assess whether adoptive transfer of sensitized T cells induced any neurologic toxicity over a longer period of time. Patients were vaccinated with irradiated autologous tumor cells mixed with GM-CSF approx 1 mo after completion of standard radiation treatment. The

yield of LN cells for these patients ranged from 9×10^7 to 1.1×10^9 and was approximately four times greater than what was previously obtained in the cohort of patients with recurrent GBM. Minor modifications to the procedure of ex vivo stimulation but with retention of a low concentration of IL-2 (10 U/mL) resulted in a median of 42-fold proliferation of cells over 6–8 d. These culture conditions permitted delivery of a median of 1.1×10^{10} cells that were predominantly CD4+ and nearly uniformly CD25+. The T cells were administered as outpatient therapy, without concomitant IL-2, and there was no immediate toxicity. Interestingly, four patients had partial regression of measurable residual enhancing tumor masses without development of immediate or delayed neurologic toxicity. In addition, three patients with minimal residual tumor have been free of tumor progression for greater than 2 yr (103).

Similarly designed phase I clinical studies were performed in patients with metastatic RCC or head and neck squamous cell carcinoma (104,105). These studies used T cells alone without concomitant IL-2 and were performed entirely as outpatient therapy. The extent of numerical amplification of T cells and retention of a predominance of CD4+ T cells was similar to the previous experience with glioma patients, indicating that there was not a significant restriction of this approach to a single disease. There was no significant toxicity from the T-cell transfer. Among 20 patients with progressive metastatic RCC, 1 had a partial response, 1 a mixed response, and 8 had stable disease lasting greater than 5 mo. These results indicated that further modifications to improve the function or to sustain the T cells were needed before more extensive clinical testing was warranted.

6. CONCLUSIONS

Adoptive transfer of tumor-sensitized T cells can mediate the regression of established tumors in all tested anatomic sites. Animal models have established critical parameters such as the optimal sources of T cells, kinetics of in vivo sensitization, and phenotype of the tumor-reactive subset. The importance of effective methods of ex vivo stimulation, as well as variable effects of concomitant treatments such as systemic IL-2 on trafficking and effector function have been extensively tested in preclinical models. It is evident that CD4+ and CD8+ T cells as well as host APCs and macrophages participate in effector mechanisms. Therefore, methods to stimulate and preserve a broad repertoire of T cells and augment the function of macrophages will be desirable for clinical applications. Clinical testing of adoptive immunotherapy with autologous tumor-sensitized T cells is still at an early stage but has so far demonstrated a remarkably low degree of toxicity relative to other accepted forms of cancer therapy. Autoimmune reactions are a potential concern especially as the efficacy of the therapy is increased through improvements in cell culture techniques and intensification of host conditioning. In some studies reactivity against normal melanocytes has led to vitiligo in some patients but this may reflect a consequence of using tissue-restricted rather than tumor-specific antigens. Future improvements in tumor vaccines for T-cell sensitization and methods to purify tumor-reactive T cells prior to culture activation and promote the function and persistence of transferred T cells, will broaden clinical applications of adoptive immunotherapy for cancer.

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III

TUMOR-SPECIFIC VACCINE DEVELOPMENT

Jeffrey Weber, MD, PhD

CONTENTS

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1. INTRODUCTION

The recent clinical experience with melanoma vaccines reflects a historical dichotomy. Current phase III trials have employed cellular vaccine strategies developed in the 1970s and have shown some hints of clinical benefit, whereas less developed but more rigorously derived strategies employing peptides, viral vectors encoding peptide sequences, and dendritic cells pulsed with peptides are still undergoing proof of concept phase I and II pilot trials and will not advance to the point of randomized phase III trials for some years to come. Over the next 5 yr, several large randomized cell vaccine trials will mature as we pursue newer ideas with peptide vaccines for melanoma based on molecular genetics and immunology. In this chapter I will describe recent evidence suggesting that a cell vaccine may generate a class I-restricted immune response to melanoma antigen peptides and may have utility in the adjuvant therapy of intermediate- and high-risk melanoma. This is followed by a description of the some of the most promising of the multitude of new peptide vaccine approaches available to patients with resected and metastatic melanoma today.

2. CELLULAR VACCINE APPROACHES

The vaccine that has had the widest testing and has been the subject of the largest randomized trials to date is Melaccine, a lysate of two melanoma cell lines developed by Malcolm Mitchell in the 1970s that is lyophilized and delivered with an adjuvant called

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DETOX. DETOX consists of monophosphoryl lipid A from *Salmonella minnesota* and a mycobacterial cell wall skeleton in an oil-in-water emulsion (1,2). An 8% response rate was observed in several early phase I and II Melaccine trials comprising a total of 139 patients with stage IV melanoma with a 20-mo median survival, although 10 of those patients had no measurable disease. A phase III study was conducted in 112 patients with stage IV melanoma who were randomly assigned to receive weekly Melaccine or the four-drug regimen of DTIC, Cisplatin, BCNU, and Tamoxifen for two 28-d cycles. The response rate for Melaccine was again 8%, and median survival was 12.3 mo for chemotherapy compared with 9.4 mo for Melaccine ($p = 0.16$), demonstrating no significant difference between the arms. Toxicity was considerably lower in the vaccine group, suggesting that quality of life might be improved in that arm (3). Several anecdotal cases have been published suggesting that Melaccine was capable of inducing responses in patients with metastatic melanoma (4). Based on these data, in 1992 the Southwest Oncology Group initiated a trial (S9035) of 2 yr of Melaccine compared with observation for patients with localized, intermediate-risk melanoma (primary lesions 1.5–4 mm in thickness, node-negative, stage IIA). With a median follow-up of 4 yr and median of 5.6 yr for surviving patients, the results for all 689 randomized patients show no significant difference in relapse-free survival (5-yr RFS 66% for vaccine arm vs 62% for observation, $p = 0.17$), with 65% of patients alive at 5 yr. Twenty-four percent were surgically staged, but the remaining patients were clinically node negative (N0). Eighty-nine patients (13%) were deemed ineligible. Toxicity was mild to moderate in most patients receiving the vaccine, but 9% had Grade III toxicity. These data suggested that for all patients as randomized, Melaccine did not demonstrate a clinical benefit (5).

Prior to the initiation of the S9035 trial, it was shown that 12 of a select group of 98 stage IV melanoma patients who received Melaccine in different phase I and II trials had evidence of partial and complete response or prolonged stable disease (6). An analysis of the human leukocyte antigen (HLA) class I typing of those patients was performed to determine whether there was an effect of haplotype on clinical course. Only 70 of the 98 had HLA data available, including all 12 who demonstrated clinical benefit. A significant association was seen between two or three of the following five HLA class I alleles and clinical benefit: A2, A28, B44, B45, and C3. The strongest association was observed with A2 and C3 expression. These preliminary data in a limited number of highly selected patients suggested that a class I-restricted T-cell response might be associated with a beneficial response to Melaccine. Therefore, soon after the S9035 trial began, the protocol was amended to include HLA class I typing for all consenting patients in order to determine whether there was an association between expression of any of the five alleles postulated to confer clinical benefit to vaccination with Melaccine and relapse-free and/or overall survival in the vaccinated group. Those data have been published (7). Based on these provocative data S0201, a prospective randomized phase III trial of 2 yr of Melaccine vs observation for 950 intermediate-risk patients with pathologically staged IIA, IIB, and IIIA (N1a) melanoma who are HLA-A2 and/or -C3 will be conducted. There was a very significant difference in disease-free (and overall) survival for the subgroup of patients that were HLA-A2 and/or -C3 positive, comprising 58% of the total, as predicted from the prior study of 70 selected patients from phase II trials published in 1993 (5,7). The relapse-free survival curves for patients that were A2/C3 indicating that there was a significant prolongation in the RFS in those patients only in the vaccinated

group, $p = 0.004$, compared to observation patients, or those who were not A2/C2 positive. This was true if patients were A2, or C3, or both. These data indicate that both retrospectively and prospectively there appeared to be clinical benefit for patients who are HLA-A2 and or -C3 positive and received Melaccine. It would appear that a class I-restricted phenomenon had occurred, consistent with recognition of class I-restricted peptide antigens by A2/C3 patients receiving Melaccine that led to clinical benefit (8). Either antigenic peptides in the Melaccine lysate were being processed and cross-presented by host antigen-presenting cells (APCs), or intact peptide–MHC (major histocompatibility complex) complexes were directly presented to T cells in the host. The former alternative is overwhelmingly likely, since not all of the five associated HLA antigens that conferred benefit in the phase II analysis were present on the Melaccine cell lines, HLA-A2 is not detected at all on the Melaccine cell lines, and it is quite unlikely that intact class I/peptide/membrane complexes survive the process of preparing the lysate. These data suggest that the mode of action of a cellular vaccine is actually mediated through recognition of melanoma antigen peptides. A follow-up trial is planned for those patients that are HLA-A2 and/or -C3 who will receive Melaccine for 2 yr or be observed.

Another vaccine based on allogeneic cell lines is CanVaxin™ (previously called CancerVax), a mixture of three allogeneic cell lines administered with bacille Calmette-Guérin (BCG) as an adjuvant. It has been tested in a number of phase II single-arm trials in different scenarios. In one trial, 150 patients with resected stage III or IV disease were included. The BCG was given by scarification for the first two vaccinations, which was then administered for 2 yr. Of the patients who received CanVaxin, the overall survival at 5 yr was 39%, compared with the nonrandomized but matched group of 113 patients who has a 19% 5-yr survival rate (9). Overall survival appeared to correlate with delayed-type hypersensitivity (DTH) skin reactivity to the vaccine.

In a trial that included 83 patients with resected stage II melanoma, serologic reactivity by generation of immunoglobulin M (IgM) antibodies to a 90-kDa glycoprotein contained within CanVaxin appeared to correlate with prolonged disease-free and overall survival (10).

A total of 54 patients with in-transit metastases were treated with 2 yr of CanVaxin (11). There were seven complete responses (CRs) and two “regressions” for an overall response rate of 17%. The median duration of response was 22 mo, with median overall survival of 53 mo. Of 25 relapses, 23 were retreated with vaccine with a median survival of 14 mo. For all patients, survival correlated with vaccine-induced DTH. Clinical benefit as defined by survival or tumor response with immune assays was demonstrated was shown to be correlated with DTH to the cell lines in CanVaxin and development of an IgM (but not IgG) antibody response to a 90-kDa immune complex called TA-90. In one group of 76 patients, anti-TA-90 IgM correlated with overall survival with $p = 0.051$, and higher IgG actually correlated with worse outcome. Median survival was found to be 76 mo with high IgM and high DTH to CanVaxin, compared with 13 mo if neither were elevated.

The TA-90 marker discovered as a correlate of CanVaxin-induced clinical benefit was analyzed for its prognostic effects in 219 patients with resected stages III and IV disease (12). A total of 47% of 219 patients converted from TA-90 negative to positive during vaccination, which correlated with favorable survival, 78% vs 63% at 2 yr, with $p < 0.02$. These intriguing data suggest that CanVaxin may be beneficial, although the shortcomings of retrospectively controlled nonrandomized phase II comparisons lend caution to

the interpretation of those data. In order to test the notion that CanVaxin is a useful clinical reagent, it is being tested in a large 800+ patient randomized trial that is now ongoing in patients with resected stage III melanoma. The success or failure of that placebo-controlled, well-powered phase III study will determine whether CanVaxin will enter the melanoma vaccine armamentarium. The utility of the TA-90 marker is still unclear, and it is puzzling that although the vaccine-induced induction of TA-90 titers is favorable, endogenous TA-90 levels are not.

A different approach to an allogeneic vaccine has been the development of a cell-free shed polyvalent vaccine derived from multiple melanoma cell lines. It is highly likely that the shed vaccine is composed of melanoma peptides. A small phase III trial of 38 patients with resected stage III melanoma was carried out in which 24 patients received the vaccine for a duration of 12 mo, then every 6 mo until progression or death compared with 14 who observed (13). Survival was prolonged in the small vaccinated group with $p = 0.04$, to 3.8 yr in the vaccinated group compared with 2.7 yr in the observed group. Overall survival correlated with tumor cell supernatant DTH reactivity. Bystryn and colleagues summarized results from 31 patients treated with the shed polyvalent vaccine in which extensive immunologic analyses of immune reactivity in fresh blood samples were performed (14). They found that 17 of 20 strongly HLA-binding epitope peptides were recognized by peripheral blood melanoma specific T cells derived from immunized patients, indicating that their shed vaccine induced a broad peptide-specific immune response, and that a larger variety of antigen peptides than previously thought were capable of eliciting an immune response in patients. Interestingly, there was a profound patient-to-patient variation in ELISPOT response to different peptides, with no evidence seen of a response to any HLA-A3-, -A11-, or -B7-restricted immunodominant peptide. This approach is being considered for a large randomized trial.

3. PEPTIDE VACCINE STRATEGIES

Over the last decade, at least 20 HLA class I-restricted peptide epitopes have been defined from multiple melanoma differentiation and cancer-testis antigens. They are reviewed in ref. 15. Peptide vaccine trials have been conducted with individual or multiple peptides in patients with stage IV disease in aqueous solution or using different adjuvants, with different cytokines, or via DNA plasmid delivery. Some trials have already been conducted in high-risk resected patients, which would appear to be an optimal population for conducting cancer vaccine trials. Significant controversy exists, however, over the optimal surrogate immune end point to measure in peptide vaccine trials, appropriate dose, the duration of treatment, and scheduling.

The first tumor antigen recognized by T cells that was cloned was MAGE-1, a member of a multigene family dubbed a “cancer-testis” antigen because it was found to be encoded normally in the germline but expressed only in tumor tissue, testis, and placenta (16,17). Since the testis was regarded as an immune sanctuary due to lack of class I expression on its parenchymal and germ cells, the MAGE genes appeared to constitute an optimal group of tumor antigens for vaccine strategies because of their preferential expression by tumors. Clinical trials of an HLA-A1 peptide encoded by MAGE-3, present on 70–80% of metastatic melanomas, resulted in evidence of tumor regression in 6 of 25 patients that received a monthly regimen of peptide in aqueous solution. Surprisingly, no evidence of antigen-specific T cells could be detected when standard techniques such as limiting dilution

analysis, ELISPOT, and tetramer assays were used (18). Only after multiple restimulations ex vivo could reactivity to MAGE-3 be detected in patients' peripheral blood. In one study by Coulie, MAGE-3 tetramers were used to detect 1:40,000 circulating CD8+ T cells with a monoclonal T-cell receptor in a patient that received repetitive vaccinations with the MAGE-3 peptide alone (19). In another trial of the same MAGE-3 epitope administered with incomplete Freund's adjuvant (IFA) to patients with resected melanoma, modest levels of immunity were seen in 5 of 18 patients, with functional lytic T cells detected in circulating peripheral blood mononuclear cells (PBMCs) (20).

The definition of a number of differentiation antigens expressed by both melanocytes and melanomas such as MART-1, tyrosinase, gp100, TRP-1, and TRP-2 followed the cloning of the MAGE genes (21–28). Surprisingly, endogenous T-cell reactivity to MART-1 could be detected in a significant minority of patients with melanoma, but not normals (29,30). Clinical trials of an immunodominant MART-1 epitope peptide comprising amino acids 27–35, or its amino acid-substituted counterpart amino acids 26–35 (27L) have been conducted with peptide in aqueous solution or with adjuvants like QS-21 or Montanide ISA 51, equivalent to the IFA used in mice. The Rosenberg group demonstrated that the immunodominant epitope with IFA-induced immune responses by a restimulation assay in the majority of patients, but without tumor regression (31,32). The substituted epitope was clearly more potent as an immunogen in vivo (32). Romero and colleagues have conducted extensive in vitro and in vivo testing of MART-1 peptides and have demonstrated that immune responses by ELISPOT and tetramer could be detected in the majority of patients in peripheral blood; that draining lymph nodes harbored large numbers of MART-1 antigen-specific cells that were antigen-experienced classic memory-effector cells; that T-cell receptor populations after vaccination were not focused but were quite diverse; and that occasional tumor regression resulted from vaccination with MART-1 peptides in aqueous solution or with adjuvants (33–37). Weber and colleagues have shown that in patients with resected stages III/IV melanoma at high risk for relapse, the MART-1 immunodominant epitope with IFA-induced immune responses in the majority of patients that correlated with time to relapse, suggesting that clinical benefit might be associated with MART-1 vaccination (38).

Rosenberg and colleagues have shown that an immunodominant gp100 epitope 209–217 modified by the substitution of an amino acid at the second anchor position for class I was more effective at generation of an antigen-specific T-cell response in vitro and in vivo (39). The substituted epitope peptide 209–217 (210M) with Montanide ISA 51 was immunogenic in virtually all HLA A *0201 patients, and in vitro and in vivo was superior to the native peptide at the generation of immune responses. When the substituted epitope peptide was administered with interleukin-2 (IL-2), regression of tumor in a high proportion of patients was observed (40). The T-cell repertoire after gp100 immunization was found to be diverse and oligoclonal, and the kinetics of gp100-specific T-cell responses indicated that increases were seen after each vaccination, with a plateau only after multiple vaccinations and an increase in T-cell receptor avidity over time after multiple vaccinations (41–44). Significant levels of gp100-specific circulating T cells were achieved, although there did not appear to be a correlation between detection of functional T cells and tumor regression (45). Surprisingly, only 1 of 11 patients treated with gp100 peptide/IFA with IL-2 had detectable tetramer-positive antigen-specific T cells, compared to six of six with the adjuvant IFA alone (46). The gp100-specific T cells over time were found to be antigen-experienced, functional, effector-memory T cells that were

CCR7-, CD45RA+, CD44+, and CD27+ (47,48). After gp100 vaccination, there was evidence of epitope spreading to the cancer-testis antigen MAGE-12, indicating that indirect mechanisms for tumor regression might occur. Large numbers of antigen-specific T cells were found to infiltrate tumors after peptide vaccination (49), even though the levels of circulating cells varied enormously, suggesting that an assessment of the tumor milieu was probably important for any evaluation of an antigen peptide vaccine strategy. That same group found that the substituted gp100 209-217 (210M) and 280-288 (288V) peptides were potent immunogens, and that tumor-infiltrating lymphocytes that were therapeutically effective recognized those gp100 epitopes. That group isolated tetramer-positive antigen-specific T cells and found that gp100-specific effector cells from the fresh blood were able to produce interferon (IFN)- γ after exposure to antigen in the form of peptide- or antigen-expressing tumor cells, and that there was a correlation between intensity of tetramer staining and production of IFN- γ by cytokine flow cytometry or quantitative RT-PCR assay (43). In patients with metastatic melanoma who had a good immune response to the gp100 peptides and survived 2 yr after being vaccinated, antigen-specific effector cells could still be found in the bloodstream (44). In another trial, effector-memory T cells that were detected by functional ELISPOT assay were found to be highly conserved in patients with resected stages III or IV melanoma over a period of 1.5 and 3 yr after finishing a 6-mo vaccination regimen. Their phenotype was CCR7 negative, CD45RA positive, CD44 positive indicating that they were activated effector-memory T cells (Chiong et al., manuscript in preparation). Similar data have been observed by the Rosenberg group for patients with metastatic melanoma, although in their assays, there was no correlation between increased frequency of vaccine-induced gp100-specific T cells by tetramer assay and regression of tumor, although tetramer-positive cells tended to also be γ IFN producers (42).

The same substituted gp100 peptide has been used in combination with a tyrosinase peptide, and with the MART-1 immunodominant 26-35 (27L) epitope with different cytokines in patients with resected melanoma who received a prolonged series of vaccinations. Weber and colleagues demonstrated that IL-12 administered with a multipeptide vaccine was an effective immune booster (50), as was granulocyte-macrophage colony-stimulating factor (GM-CSF). Approximately 80–90% of patients had augmented antigen-specific immunity by tetramer and cytokine release assays, and the time to plateauing of the immune response was quite prolonged, with six to eight vaccinations required for optimal immunity, in agreement with findings by the Rosenberg group. In the trial of gp100/tyrosinase peptides with IFA and GM-CSF, epitope spreading against MART-1 was observed (51), confirming the prior finding that an expanded T-cell receptor repertoire may be important for clinical benefit.

The gp100 280-288 peptide has also been shown to be immunogenic. Slingluff and colleagues have immunized 22 patients who had resected stage III or IV melanoma with that peptide with or without a tetanus epitope peptide. Of 22 patients, 79% had T-helper responses to tetanus, but only 14% to the gp100 280-288 epitope (52). That same group has also demonstrated that high levels of gp100-specific T cells may be isolated from the lymph nodes draining vaccine sites, which might be a surrogate marker for the success of a peptide vaccine (53). A variety of factors may affect the ability to mount an immune reaction directed against class I-restricted peptide vaccines. Class I HLA antigen A2 is the most common alleleotype in the United States, comprising 46% of the population. The

locus is quite polymorphic, with at least 12 A2 alleles, of which A*0201 is the most common in the United States. Marincola and colleagues found that different HLA-A2-restricted melanoma peptides bound with differing affinities to A2 alleles, so that the immunodominant gp100 and MART-1 epitope peptides were recognized by PBMCs from patients expressing the A*0201 allele but not A*0204 or A*0207, for example (54). A vaccine that might work effectively in a Northern European population that was predominantly A*0201 might not work well in Southern Europe where other alleles may be common.

Many of the defined melanoma epitope peptides are derived from differentiation antigens like MART-1, tyrosinase, or gp100, which are both heterogeneously expressed on many tumors or simply downregulated in many metastatic lesions. The lack of expression of those antigens on more advanced tumors suggests that earlier-stage tumors may be more effectively targeted by peptide immunization strategies, and also that the use of multiple epitopes in a vaccine would diminish the chance of immune selection resulting in tumor variants that could escape recognition. There are contradictory data on this issue. In one study, the majority of relapsing patients continued to express the antigen (gp100) against which they were vaccinated (51). In several other studies, relapsing patients' tumors were shown to lose expression of melanoma antigens with which they were immunized (55,56). In one study, MART-1-specific T cells were shown to correlate with spontaneous regression of primary melanomas, and when tumors progressed after regression of the primary, loss of MART-1 expression was observed, consistent with an immune selection hypothesis and the idea that MART-1 is a tumor regression antigen (57). Melanomas have also been shown to downregulate or completely delete expression of class I MHC or β -2 microglobulin, which could also result in the phenomenon of immune escape (58).

Further evidence for the idea that MART-1 and gp100 are true “tumor regression” antigens comes from the demonstration that tumor-infiltrating lymphocytes (TILs) often recognize those antigens. In one study, 19 of 59 TIL cultures recognized known immunodominant MART-1 or gp100 peptides (59). The group of Jager and Knuth has conducted several multipeptide vaccine trials in patients with metastatic melanoma, and have demonstrated that tumor regression in a small trial with GM-CSF administered as an adjuvant cytokine correlated with antigen-specific immune responses (60). The same group has also intensively studied T-cell responses to MART-1 and has shown that development of a clonal T-cell response to that antigen correlated with tumor regression, strengthening the idea that a successful peptide vaccination conferred clinical benefit (61).

Immunization with melanoma epitope peptides might result in a “bystander” reaction in which the phenomenon of epitope spreading occurs. Reactivity to epitopes not included among those in the vaccine have been demonstrated for MART-1 (51) after vaccination with gp100 and tyrosinase epitopes, and for MAGE-12 after a gp100 vaccination (47). This might expand the number and type of antimelanoma T cells, and provide clinical benefit.

Tyrosinase encodes a number of HLA-A2-restricted epitopes, and several have been shown to be immunogenic in patients. The tyrosinase 1-9 and 368-376 (370D) peptides have been used to vaccinated patients with stage IV melanoma, and modest levels of reactivity were observed by ELISPOT assays. The 368-376 (370D) peptide is unique in that it results from a post-translational modification to the 370 amino acid, and is actually

expressed on tumor cells. Lewis and colleagues immunized nine patients with the modified tyrosinase 368-376 peptide with adjuvant QS-21, and found that only two of nine had an augmented frequency of cytolytic T cells (CTLs), without evidence of clinical benefit (62). Scheibenbogen immunized 16 patients with the tyrosinase 1-9 epitope, and an additional 2 patients who had no evidence of disease (NED) (63). The peptides were administered in aqueous solution with GM-CSF. Only five patients were able to receive six injections over 14 wk. One mixed response and two patients with stable disease were seen. There was evidence of augmented tyrosinase-specific T-cell immunity by ELISPOT assay in four patients, including the one patient with a mixed response, one patient with stable disease, and the two patients who were NED.

Several trials have demonstrated that clinical benefit correlated with peptide-specific immunizations. Keilholz and colleagues reported that several patients who had a history of frequent relapses of stage IV melanoma received an extended series of immunizations with a tyrosinase peptide and adjuvant and experienced long-term freedom from recurrence while levels of circulating immune cells were detected against tyrosinase (63). Jager has shown that three patients in a small series immunized with multiple peptides derived from differentiation antigens with GM-CSF as an adjuvant had tumor regression, which correlated with immune response and DTH reactivity, as have other groups (60,61).

Another member of the “cancer-testis” family is NY-ESO-1, a protein that is highly expressed on a variety of epithelial carcinomas and melanomas. It is unique among the known melanoma antigens because it encodes antibody, T-helper, and cytolytic epitopes (65). In fact, it has been shown that CTL responses correlated with the detection of endogenous antibody responses in patients with NY-ESO-1 expressing melanomas, suggesting an interdependence of T-helper responses and CTL immunity (66). Three different class I HLA-A*0201-restricted epitope peptides have been tested in a small pilot trial in 12 patients with metastatic melanoma (67). Seven of 12 patients had no detectable preexisting NY-ESO-1-specific antibody response. Of those patients, four had increases in CTL reactivity, which correlated with disease stabilization or regression. Interestingly, that same group (68) immunized patients with the NY-ESO-1 157-167 epitope and noted that patients exhibited 159-167 specific response but that epitope was not naturally expressed by tumor cells. The 157-165 peptide is the “natural” epitope, yet was a minor component of the NY-ESO-1 reaction, indicating that patterns of peptide-specific reactivity were unpredictable.

Class II peptides have been defined from a number of melanoma antigens, were tested in transgenic mice and other *in vitro* systems, and found to be capable of generating immune responses *in vitro* (69–72). These peptides are restricted to common class II alleles such as DRB*04 01, or DP4, representing 25% and nearly 50% of the population, respectively. There is evidence that class II antigen-specific peptides might add to the ability to generate a class I-restricted CTL response in melanoma, and several trials are pursuing the idea of combining class I and class II peptides in a melanoma vaccine strategy (73).

The development of careful immunologic monitoring for melanoma peptide trials is critical for the success of the peptide vaccine approach. ELISPOT assays have been used by a number of investigators for cancer vaccine trials and for HIV vaccine studies. This assay is technically demanding and requires dedicated equipment, but provides a reliable functional and quantitative assay for the detection of circulating antigen-specific T cells

(74–76). Cytokine flow cytometry (CFC) is a technique that permits an assessment of function and permits enumeration of antigen-specific cells, but is not as well developed or as sensitive as the ELISPOT assay (77). The CFC assay has enormous flexibility to incorporate multiple markers of immune function, and will continue to be refined for future trials. The peptide-tetramer assay is a flow cytometry-based assay that provides great sensitivity, reliability, and quantitation, but is not a functional assay (78,79). In fact several groups have described a dichotomy between the enumeration of circulating cells by tetramer assay and the number of cells detected by the functional CFC or ELISPOT assays, which reinforces the notion that a quantitative and a functional assay are critical for proper interpretation of immune monitoring of a peptide vaccine trial (80).

The most extensive and convincing data for the generation of long-lasting melanoma-specific immunity exist for patients treated on peptide vaccine trials. There has been little toxicity to those approaches, but evidence is compelling for the detection of significant augmentation in levels of antigen-specific CTLs in the circulation, in lymph nodes draining vaccine sites, and even infiltrating tumors. Long-term immunity with the existence of effector-memory T cells lasting years after vaccination has been clearly shown. IL-12 and GM-CSF injected locally are likely to be potent adjuvants, and long duration of immunization is likely to be needed. There is no indication that a dose response above 1 mg exists for peptide vaccines. The antigen-specific CTLs induced after a peptide vaccination are a mixture of low- and high-affinity effectors, and reflect a diverse repertoire of T-cell clones. The factors that determine why very different levels of immunity with a range of 1000-fold are generated in different patients remain to be determined, and are an obstacle to the further success of peptide vaccine strategies. The importance of class II peptides and their role in facilitating class I immunity remains to be defined.

4. DENDRITIC CELLS AS PEPTIDE VACCINE ADJUVANTS

Steinman and colleagues have established the dendritic cell (DC) as the most potent APC for the generation of naïve and restimulated T-cell responses. Promising preclinical work in mice rapidly led to clinical trials of DCs pulsed with peptides and/or tumor lysates. Nestle and colleagues (81) found that intranodal injection of peptide- and lysate-pulsed DCs resulted in a 25% response rate in an initial trial of 16 stage IV melanoma patients. Immune responses were observed in clinical responders, some of which were quite durable. Schuler et al. have employed a variety of peptides for DC pulsing (82), and showed that mature DCs pulsed with a MAGE-3 HLA-A1-restricted peptide were capable of inducing transient mixed responses in heavily pretreated patients. In 8 of 11 patients, MAGE-3 specific CD8+ CTLs were detected in the circulation (83). The same group found that cryopreserved DCs pulsed with class II peptides injected subcutaneously induced clear-cut immunity against class II DP4- and DR13-restricted peptides by a careful ELISPOT analysis in the majority of patients, and that in some cases tumor cells sharing those alleles were recognized. Minimal responses were seen clinically, however (84). In a different approach, PBMCs were simply pulsed with MAGE-3 or MART-1 peptides and injected intravenously with recombinant IL-12. Six of eight patients with boosted immunity had evidence of tumor regression, including one CR and one PR, one minimal response and six with stable disease (85).

Banchereau's group has pioneered the use of DCs derived from CD34+ stem cells. Immature DCs were matured in the presence of tumor necrosis factor (TNF)- α , pulsed

with multiple melanoma peptides and injected intravenously in 14 melanoma patients. Two partial responses were seen, and immune manifestations included vitiligo in one patient, development of class I-restricted T-cell responses in one patient, and DTH skin test positivity to melanoma antigens in four patients (86). A controversy exists over the optimal type of DC to use as an immune adjuvant, since immature DCs, which are able to phagocytose and take up antigen but may not optimally present antigen to T cells may not be the most physiologic APC. Mature DCs, which have lost the ability to phagocytose exogenous antigens, have upregulated levels of costimulatory markers and MHC molecules and are well adapted for antigen presentation. Jonuleit addressed the issue of the “optimal” DC by comparing immature and mature DCs in a small pilot trial. Mature DCs were clearly capable of promoting a greater level of CTL and T-helper reactivity measured in the circulating PBMCs (87).

DCs have been injected intranodally resulting in clinical responses, as shown above, but Figdor’s group injected radioactively labeled DCs intranodally and found that they were capable of migrating to T-cell areas of adjacent nodes, a favorable feature (88). Morse et al. had previously labeled DCs to perform trafficking studies demonstrating that intravenously injected DCs tracked to spleen, liver, and lungs but not nodal regions; subcutaneously injected DCs mostly resided at the injection site in the soft tissues; and intradermally injected DCs had the highest likelihood of migrating to draining nodal areas, but only a very small percentage of the cells actually migrated away from the area of injection (89). Those authors concluded that the intradermal route would result in the most efficient delivery of DCs to nodal draining areas.

Viral vectors have been used to transduce DCs in order to express tumor antigen peptide epitope sequences, whole-protein sequences, and cytokine molecules. The use of a vector that may encode many different epitope peptides would minimize the chance that antigen downregulation could permit antigen-escape variants to avoid immune recognition.

What can one conclude from these peptide-pulsed melanoma DC data? A very modest number of clinical responses have been observed with antigen-pulsed DCs; mature DCs are probably more clinically and immunologically effective than immature DCs; the intradermal and intranodal routes are probably best; the optimal way to deliver antigens via the use of DCs has not been well defined; and finally it is unclear that the use of DCs, with all their attendant complexity, expense, and risk of contamination, is any better than a number of other technologies for antigen delivery.

5. OTHER APPROACHES

The use of purified gangliosides, which are described in detail in Chapter 11, has been pursued in a large randomized clinical trial in patients with stages IIB and III resected melanoma at very high risk for relapse. Patients were randomly allocated to receive the 1-yr high-dose IFN- α -2b regimen or 20 mo of a GM2-KLH vaccine with adjuvant QS-21. The trial was halted by the data safety-monitoring committee before accrual of all patients since a predetermined difference in RFS favoring the interferon arm with a *p* value of 0.01 was observed (90). Although somewhat immature, with a median of approx 2 yr of follow-up, these disappointing data make it extraordinarily unlikely that the GMK ganglioside vaccine would be equivalent to interferon as treatment for resected high-risk melanoma. The addition of the GMK vaccine to interferon might be a desirable option,

since in a small pilot trial, concurrent IFN did not diminish the generation of immunoglobulin G (IgG) antibody titres to the GMK vaccine (91).

In more novel, high-risk approaches, Parmiani et al. (92) isolated heat shock proteins (HSPs) from 39 patients with stage IV melanoma, and injected them intradermally. Of 28 patients with measurable disease, there were 2 CR and 3 stable disease (SD). Tumor antigen-specific T-cell responses were observed in both CR patients by ELISPOT assays, and in 11 patients overall. The tumor-specific HSPs carry tumor antigen peptides that have been shown to confer their immunogenicity. Tumor-derived exosomes or exosomes derived from DCs loaded with tumor antigen have been studied in a phase I trial in Europe (1). The treatment was well tolerated, and the exosome vaccine was prepared from a single leucopheresis for 11 of 12 patients. A mixed response was observed and one patient had stable disease. This approach is being pursued in a phase II trial in the United States. DNA plasmids encoding multiple melanoma peptides from the antigen tyrosinase have been developed in which intranodal infusion was achieved via a pump over 96 h with minimal toxicity. Several tyrosinase epitope sequences were incorporated into the plasmid design, but immune responses by tetramer assay were only observed to the 207-216 epitope; interestingly, although no clinical responses were observed, immune response to tyrosinase was correlated with prolonged survival (Tagawa et al., manuscript submitted).

A completely novel approach that takes advantage of a significant body of murine work is that of the NCI group (94) led by Rosenberg, which treated patients with a modification of their TIL procedure in which the adoptively transferred cells were infused into patients who had been rendered lympho-neutropenic by Fludarabine and Cytoxan pretreatment, followed by high-dose IL-2. The concept being tested was that after being rendered lymphopenic, there was a “re-setting” of the patients’ lymphocyte homeostatic mechanism and there could be a selective expansion of the adoptively transferred cells to the “empty” lymphoid compartment. In fact of 13 patients treated, there were 6 partial responses (PRs) and 4 stable patients with some patients demonstrating 80–90% circulating adoptively transferred cells that exhibited T-cell receptor clonality by molecular analysis. Ten of the 13 TIL cultures that were adoptively transferred were gp100 or MART-1 melanoma peptide-specific. This is a promising approach with implications for peptide immunization strategies, and should be pursued further.

6. CONCLUSIONS

A varied number of approaches have been taken to augment immunity in patients with melanoma, and small numbers of highly selected patients with metastatic disease have been shown to have regression of melanoma after receiving peptide vaccine therapy, but no melanoma vaccine currently appears to have promise as a first-line therapy for stage IV melanoma. Cell vaccine approaches are in advanced phase III trials and the two leading “contenders,” Melacine® and CanVaxim™, each have preclinical evidence using surrogate markers to suggest that they might be beneficial in the adjuvant setting after resection of high-risk disease, and Melacine appears highly likely to induce melanoma peptide-specific T-cell responses. Carefully performed trials of 800–1000 patients with peptide-specific correlative studies will be necessary to define the utility of the cell vaccine approach, which appears to have limited adaptability and little room for improvement.

Antigen-specific peptide vaccine approaches are less well developed, still at an infant stage of development, and present a bewildering array of possible delivery mechanisms,

adjuvants, doses, and schedules. From the peptide trials, a number of lessons stand out. Multiple vaccinations over a prolonged period will be needed to achieve high levels of immunity. Once a peptide-specific immune response is achieved, it is likely to induce the development of long-lived memory-effector T cells. Repetitive vaccinations result in T-cell receptors of higher affinity, although the T-cell repertoire remains oligoclonal. Multiple peptides restricted to different HLA alleles should be used as immunogens to avoid immunoselection or immune escape by antigen loss or downregulation of class I molecules. Metastatic disease appears to be a poor clinical scenario in which to study peptide vaccine strategies. DC approaches have captured the interest of a large number of investigators, yet for all the effort in that field, little clinical benefit has been seen in DC trials. The advantage of the antigen-specific approach, using peptides, alone, pulsed onto DCs, or expressed by viral/DNA vectors is the ability to generate high levels of tumor antigen-specific T cells, and sensitively monitor levels of antigen-specific T cells in the lymph nodes, blood, and tumors. The results observed thus far indicate that we are still far from achieving consistently high-level, clinically beneficial, and long-lasting T-cell responses with current strategies. Such basic questions as the importance of T helper cells, the need to overcome cytokine-induced immune suppression, and the ability to identify true tumor regression antigens are inadequately addressed or not answered at all. Nonetheless, we must continue to refine antigen-specific strategies, search out new antigens, and format a combined modality therapy in which elimination of immune suppression will facilitate the generation of potent and long-lasting immune responses that will bring clinical benefit (95).

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1. INTRODUCTION

Active specific immunotherapy, more commonly known as the use of “vaccines” for the treatment of cancer, has found its greatest use in melanoma. The ease of establishing primary cultures of melanoma cells, and cell lines, has enabled the study of immune responses toward melanoma more readily than most other tumors. The availability of these cell lines has facilitated the production of allogeneic vaccines for treatment.

The theoretical basis for allogeneic vaccination for cancer is the presence of antigens shared among melanomas of different individuals. Serological investigations in the 1970s (1) suggested that the use of individual-specific antigens was the most appropriate way to immunize against cancer. Shared antigens were considered “nonspecific” tissue antigens. However, as work on antigens detected by human monoclonal antibodies (2–4) and those detected by human cytolytic T lymphocytes (CTLs) (5,6) progressed it became clear that patients were reacting mainly against shared antigens, some of which were

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found in more than one histotype of tumor. Although many of the antigens are found in the normal counterpart of the tumor cells, such as melanocytes for melanoma cells, their overrepresentation on tumor cells has nonetheless made these antigens useful targets for immunotherapy.

At the same time, the impracticality of using autologous vaccines in large-scale trials became more apparent. A group of patients might have variable amounts of tumor that could be used for autologous vaccines—some, none at all—and the number of injections that could be given in a course of treatment also varied among them. Standardization of the antigens given in the vaccine preparations in a series of patients was clearly impossible, and determination of those antigens, though possible, was laborious.

The theoretical possibility that allogeneic vaccines might preempt the immune response toward alloreactivity, away from response toward the weaker tumor-associated antigens, was another initial deterrent to their use. Nevertheless, for this line of treatment to be useful to large numbers of patients, allogeneic vaccines had to be tested for their effectiveness.

Finally, the induction of autoimmunity as an inevitable side effect of active specific immunization was a concern with both autologous and allogeneic vaccines, which had to be monitored during the course of evaluating the response of the tumor.

As we hope to demonstrate here, allogeneic vaccines have proved that active specific immunotherapy has sufficient merit in melanoma to justify its further development, particularly combined with treatments aimed at the tumor's evasion or subversion of the immune response.

2. ALLOGENEIC MELANOMA LYSATES FOR THE TREATMENT OF DISSEMINATED MELANOMA

We began our studies with allogeneic melanoma lysates late in 1985, in patients with disseminated melanoma. This was done primarily for ethical reasons, because we did not know what the toxicity of this form of treatment would be. That would preclude its use in patients with minimal residual disease—the group that might best benefit from “vaccination”—until phase I data showed that toxicity was acceptable. We should note that the use of this admittedly “crude” whole-cell vaccine, together with an adjuvant that may further have obscured the effects of the tumor preparation itself, was a decision we made deliberately after some experience with extracts of autologous cells given without adjuvant—our initial attempt to use a better defined, individual-specific antigen. The failure to generate either an immune response or a clinical response in a small group of patients led us to the diametrically opposite approach: whole-cell lysates with antigens that gave the patients the choice of which to respond to, injected with a potent adjuvant to increase their immunogenicity.

For our vaccine we chose two cell lines derived from subcutaneous nodules of two different female patients, which were initiated in our laboratory in 1980 and 1981. MSM-M-1, abbreviated M-1, is amelanotic, nearly tetraploid, grows slowly, and expresses human leukocyte antigen (HLA)-A2, -B12, -B62, and -C3 and HLA class II antigens DR4, DR10, DRw53, and DQ8. It also expresses the ganglioside GD3 but not GD2. The second cell line (MSM-M-2, or M-2) is smaller, nearly diploid but with a small percentage of hypodiploid cells and trisomy at chromosome 7. It is highly pigmented, grows rapidly, expresses HLA-A28, -A31, -B51, -B60, -C2, and -C6, and is consistently devoid

of HLA class II antigens by serological assays. However, genes coding for HLA class II antigens are present. We are repeating the HLA typing by genetic methods now, because the serotyping, although performed by one of the best tissue-typing laboratories, unavoidably suffered from poor expression of HLA antigens on the tumor cells, even though we attempted to correct this by preincubation with interferon (IFN)- γ .

Lysates were produced with a Polytron stainless steel homogenizer (Tekmar Cincinnati, OH) followed in the earliest trials by freeze-thawing, purposely avoiding the use of enzymes or irradiation that we feared might reduce immunogenicity. Each cell line was lysed separately, and then mixed for the final preparation of vaccine. Examination of the lysate preparation by Ribi ImmunoChem Research, the company that was given the license to produce it after our initial trials, revealed the presence of the common melanoma antigens tyrosinase, gp100, and MART-1/Melan A, as well as gangliosides GD2 and GD3. Another common melanoma antigen in the preparation that we later discovered, which is shared also by breast and ovarian cancers, was MG 50, which we will later discuss briefly.

The immunological adjuvant in all of our trials was some version of DETOXTM produced by Ribi ImmunoChem Research. The original version of this adjuvant consisted of cell wall skeletons of *Mycobacterium phlei*, monophosphoryl lipid A ("detoxified" end toxin), squalane, and Tween-80 emulsifier. Lysates were mixed with the DETOX within 30 min of injection of the two into two sites in the arm and/or buttocks sc.

The lysates were stored frozen at -80°C in our initial trials and thawed immediately before use (which we refer to as "frozen lysates"). At Ribi ImmunoChem Research (now Corixa-Montana) a *lyophilized* version called Melaccine[®] was produced for use in multicenter phase II and III clinical trials. This form facilitated interstate shipment and storage in hospital pharmacies. Besides this difference in the lysate preparation, several different permutations of the adjuvant DETOX have been used in Ribi's successive trials, such as DETOX-PC (phosphoryl choline) and DETOX-B soluble emulsion (SE), the latest version of the adjuvant.

2.1. Initial Clinical Trials With Frozen Lysates

In our phase I and II trials (comprising 19 and 25 patients, respectively) with frozen lysates, lysates and DETOX adjuvant were given sc. Nine of the 44 patients (5 of 19 and 4 of 25, respectively) (20.5%) with measurable metastatic disease had major responses, 3 complete response (CR) (6.8%), and 6 partial response (PR) (13.6%). One additional patient in the phase II trial (2.3%) had 17 mo stability of lung nodules—developing brain metastases at that juncture—which we felt was also a response to treatment, because the lung nodules had been growing before treatment. Table 1 lists the patients who responded, the duration of response and survival, as well as their further treatment after active specific immunotherapy was discontinued. Note that 7 of 44 patients (15.9%) treated with frozen lysates in these two trials lived at least 1 yr, with a median survival in this responding cohort of 2 yr. Combining data from the phase I and II trials, the median duration of remission solely on frozen lysates was 1.3 yr. It is noteworthy that in each of these initial trials there was one responder who has survived for more than 12 yr. In the phase I trial, dose levels of 5, 10, 20, and 40 million tumor cell equivalents were tested. Clinical responses were found at all levels, but some of the most durable were elicited by 20 million tumor cell equivalents. Therefore, phase II and subsequent trials were con-

Table 1
Survival of All Responders to Frozen Lysates in Phase I and II Trials

Patient	Study	Response	Site	Response Duration	Survival	Other Treatment
LA	Phase 1	CR	SC, Lung	>17 yr	>17 yr	Surg, rad
SH	Phase 2	CR	SC, Lung	1.25 yr (PR); 6 mo (CR)	>16 yr	Surg
JB	Phase 1	CR	LN	9.5 mo	11 mo	None
CC	Phase 1	PR	SC	3 mo	1 yr	Surg
GM	Phase 1	PR	SC	2 mo	9 mo ^a	IL-2
MR	Phase 1	PR	SC, ADR, Lung	2 mo	7 mo	None
PD	Phase 2	PR	Ileum, SC	1.3 yr	2 yr	None
HJ	Phase 2	PR	LN Masses	1.75 mo	2 yr	
HH	Phase 2	PR->CR	Breast, SC	3.25 yr	4.3 yr	Rad
LR	Phase 2	SD	Lung	1.4 yr	3.25 yr	Rad

7 of 44 patients (15.9%) treated at single institution (phase I, 19; phase II, 25) lived ≥1 yr, as of 8/1/03

^aCommitted suicide during subsequent IL-2 treatment.

CR = complete response; PR = partial response; SD = stable disease (18 mo); SC = subcutaneous nodules; ADR = adrenal; Surg = surgery; Rad = radiation therapy

ducted with that dose level: 20 million tumor cell equivalents. Cyclophosphamide, 350 mg/m², was given 3 d before the first dose of vaccine to 11 patients in the phase I study, but was not given to patients in our phase II study. Most trials conducted by Ribi ImmunoChem used cyclophosphamide pretreatment, for its presumed effect of inhibiting suppressor T-cell activity (7).

2.2. Initial Clinical Trials With Melaccine

Our first phase II trial with the lyophilized Melaccine preparation, including DETOX, administered sc to 20 patients, without cyclophosphamide pretreatment, yielded an objective response rate of 15%, with 0 CR, 3 PR (15%) and 1 minor response (5%) (between 25 and 50% shrinkage of all lesions). There was also a “mixed” response (5%), not included in the group of objective responses: 33% shrinkage of a liver metastasis but >25% increase of an axillary lymph node mass, subsequently resected. This patient (DG), and his response, have lasted for more than 10 yr with monthly maintenance treatments (Table 2).

Two other trials of Melaccine lyophilized lysates given sc were performed at our institution with and without cyclophosphamide pretreatment (Table 2). Of the 58 patients entered into three trials, including the 20 described above, there were 8 major responses, 1 CR (1.7%) and 7 PR (13.8%), for a total objective response rate of 15.5%. Whether this was significantly different from initial results with our frozen lysates is uncertain. Except for one unpublished trial at a different single institution with fewer than 20 patients actually treated, there have been no direct comparisons of the two forms of the material. In any event, if we combine the data for 102 patients who received the combination of M-1 and M-2 cell lines either as frozen lysates or Melaccine in these five clinical trials, our response rate was 18.6% (19 of 102), with 3% CR and 15.6% PR. In a trial conducted by

Table 2
Patient (DG) Maintenance Treatments

Trial	Phase 2 with:	CR	PR	MR	Total
1	Melaccine alone	0	3	1	20
2	Melaccine alone	1	2	2	19
3	Cyclophosphamide + Melaccine	0	3	1	19
Total		1	8	4	58
		(1.7%)	(13.8%)	(6.9%)	(100%)

CR = complete responses; PR = partial responses; MR = minor responses (>25% but <50% decrease)

Table 3
Long-Term Responders to Melaccine

Patient	Study	Response	Site	Response Duration	Survival	Other Treatment
RB	Phase 2	CR	Lung	>13 yr	>13 yr	None
KR	Phase 2	PR	SC, LN	4 mo	3.8 yr	None
GW	Phase 2	PR	Jejunum, LN, Lung	5.4 mo	1.9 yr	IFN- α
CK	Phase 2	PR	LN	7.1 mo	2.1 yr	IFN- α
DG	Phase 2	SD	Liver		>13.1 yr	None

5 of 58 patients (8.6%) treated at single institution lived \geq 1 yr as of 8/1/03. CR = complete response; PR = partial response; SC = subcutaneous; LN = lymph nodes

Morton and colleagues with allogeneic irradiated melanoma cells and bacille Calmette Guérin (BCG), an essentially identical response rate of 23% was reported in 40 patients with disseminated melanoma, with 3 CR (7.5%), supporting our experience (8).

In all of our trials with frozen lysates or Melaccine, the sites of disease that responded best were subcutaneous, lung, small intestinal, and lymph nodes, even when extensive or massive, but bone and numerous liver lesions were far less responsive. However, shrinkage of a single metastasis, or several small metastases, in the liver was also obtained. Five of 58 patients (8.5%) treated by us with Melaccine in these initial three trials lived at least 1 yr, as shown in Table 3. Whether this represents a long-term survival that is truly lower than the 15.9% we found with frozen lysates is uncertain.

2.3. Long-Term Survival in Phase I and II Studies of Allogeneic Lysates

Four patients who responded to allogeneic lysates in our phase I and II frozen lysate studies and our initial Melaccine phase II studies—comprising three CR, and one “mixed” response—are still alive without disease >13, >13, >16, and >17 yr from initiation of treatment. This represents 4% of the 102 patients treated. A fifth patient (BP) whom we reported earlier (9) with a CR from IFN- α after failing to respond to Melaccine, is also alive at >10 yr. All but one of the patients have been free of disease, and all except the patient with the response to IFN- α has been maintained solely on the vaccine, at first frozen lysates and later Melaccine. Patient LA had a single brain metastasis removed 2 yr after her CR, and then continued NED (no evidence of disease) until 9 yr of remission, when a new, genetically different melanoma was found in her small intestine. She has now lived nearly 5 more years after induction into another remission with chemotherapy,

maintained by twice monthly courses of a combination of 1 million units of IFN- α and 7 million units/m² of interleukin-2 (IL-2), each given sc.

2.4. Multicenter Phase II and III Trials of Melaccine

A multicenter phase II trial with Melaccine conducted by Ribi ImmunoChem, involved the injection of Melaccine sc, but the subsequent phase III trials in disseminated disease and resected stage III melanoma administered it intramuscularly (im). This alteration in the route of administration was made by Ribi ImmunoChem in an attempt to minimize local granuloma formation (Von Eschen, K., personal communication, 1999). Though immunization is possible by this route, as with “naked” plasmid DNA vaccines, the location of dendritic cells in the dermis suggests that im injections may be suboptimal, and far less appropriate than intradermal or *subcutaneous* injections as a method of immunization with lysates. The preparations of DETOX differed somewhat too, from the original to DETOX-B to DETOX-PC to DETOX-B-SE.

Perhaps for these reasons, and undoubtedly many others such as the elimination of a possible selection bias on our part, the multicenter trials have given objective response rates of considerably less than we achieved: specifically, 8–10%. Nevertheless the long-term survival of responders that we observed was also noted in the Ribi phase II multicenter trial. Patients with stable disease >6 mo (23% of the group) or greater had a median survival of 23 mo (10).

In the Ribi phase III trial analyzed on an intent to treat 140 patients, but which ended well before that number was accrued due to a worldwide scarcity of DTIC, the response rate and median duration of response were the same as elicited by four-drug “Dartmouth” chemotherapy, with far less toxicity. There were 7% responders in the Melaccine arm and 10% in the four-drug arm. Interestingly, as in other trials, major responders for Melaccine had a survival of 18.5 mo, vs 15 mo for the chemotherapy, both of which exceeded the anticipated median survival duration of 8 mo in this stage of the disease. Clearly, there was an inadequate number of patients in this study to provide statistical power to discern any but a major difference between the two groups—which was not observed.

3. ADJUNCTIVE THERAPY WITH LYSATES IN RESECTED STAGE II AND III DISEASE

In 1990 and 1991, we attempted to study Melaccine with the Southwest Oncology Group in a phase III randomized trial vs no treatment in resected stage III melanoma, to see whether relapse-free and overall survival could be improved. This was at a time when the ECOG 1684 study with IFN- α -2b (INTRON-A) had not yet been completed. When the SWOG joined the Intergroup IFN- α study for stage III and deeply invasive (>4 mm) melanoma, retaining Melaccine only for resected intermediate thickness (>1.5–4.0 mm) stage II disease (SWOG 9085), our plans had to be revised. To obtain data that might support a subsequent randomized study of our own, we treated 44 consecutive patients with resected stage III melanoma from January 1992 to December 1993 over a period of 48 wk with frozen lysates + DETOX (first 17 patients) or Melaccine (final 27 patients), preceded by 350 mg/m² of cyclophosphamide. An additional 23 patients referred to us with resected stage II melanoma were also treated in a separate study by the same regimen. We began these studies as randomized trials, but quickly found that patients came to us with the expressed intention of receiving a vaccine. Those who were random-

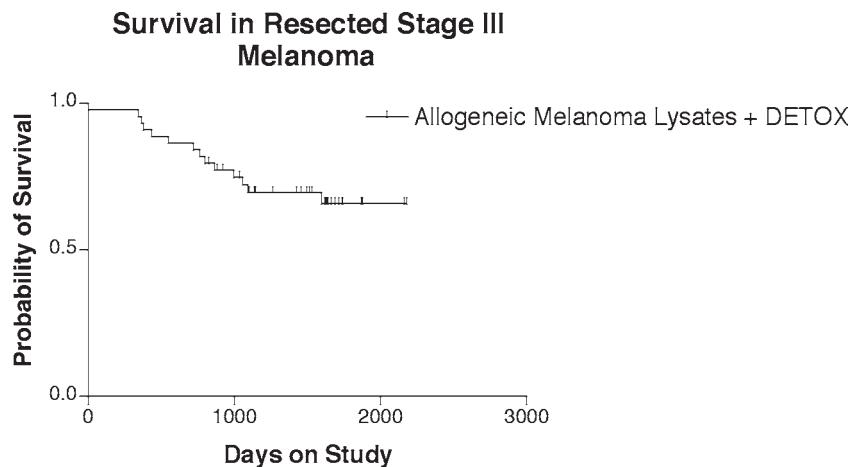


Fig. 1. Overall survival of patients with resected stage III melanoma after receiving lysates and DETOX over the course of 48 wk (Weeks 1, 2, 3, 4, 8, 16, 24, 32, and 48).

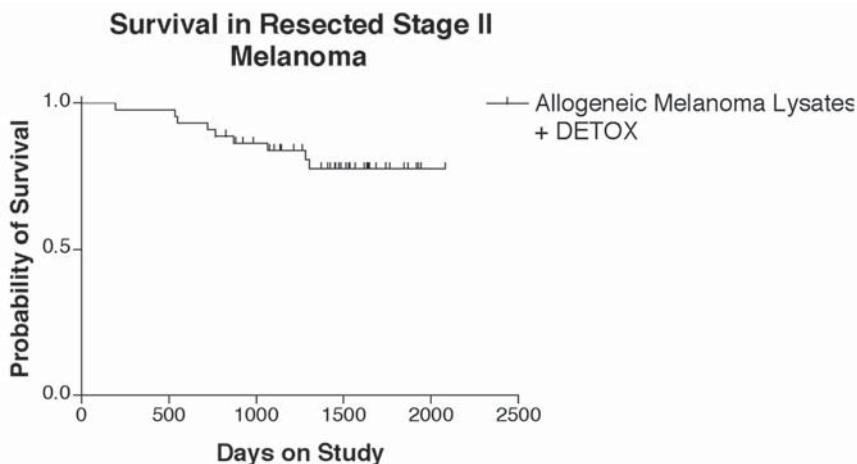


Fig. 2. Overall survival of patients with resected stage II melanoma after receiving 48 wk of melanoma lysates and DETOX (Weeks 1, 2, 3, 4, 8, 16, 24, 32, and 48).

ized to observation alone went elsewhere for immunotherapy. For that reason, we were obliged to do a one-armed study until we could set up an acceptable controlled multi-institutional trial. While unavoidably the statistical validity of our results is open to question, it is of interest that patients with either resected stage III or stage II melanoma have fared remarkably well. The trial, which was analyzed with a maximum follow-up of over 6 yr, may merit inclusion here, if only for debate over the significance of its rather striking results.

Patients were given a low dose of cyclophosphamide (350 mg/m^2) on Day 3, followed by injections of lysate vaccine on weeks 1, 2, 3, 4, 8, 16, 24, 32, 40, and 48. DETOX was usually omitted after the fourth or fifth injection when severe local granulomas appeared, in an attempt to avoid sterile abscesses. All patients were followed without further treatment until relapse and then until their death. As noted, we treated 44 patients with resected

stage III disease (median follow-up 64 mo; range 52–75 mo) and 23 patients with resected stage II disease (median follow-up 62 mo; range 51–72 mo).

The final analysis of these data was in April 1998. The results are shown graphically in Figs. 1 and 2. It is striking that the median overall survival had not been reached in either group. Sixty-six percent of the stage III patients were still alive and 46% of the group, still relapse-free. The median relapse-free survival for this group was 1104 d (36 mo). Results in the concomitant phase II trial with 23 resected stage II patients were similar, with a relapse-free survival of 1514 d (50 mo). Seventy-eight percent of the stage II patients were alive at 5 yr, and 40% were alive without relapse. Both the relapse-free and overall survivals for stage III patients considerably exceeded the expected 12–18 mo and 30–36 mo, respectively, for such patients after resection alone (11). Similarly, the overall 5-yr survival for stage II patients is usually 60–65%. In our study, the patients who relapsed with stage III disease often had regional lymph node recurrences in the resected bed rather than visceral disease, undoubtedly influencing their overall survival beneficially.

Though of course no firm conclusions can be drawn from these limited, uncontrolled studies, they were nonetheless provocative, and led to our subsequent randomized, controlled trial in resected stage III melanoma. We must caution that recent results of a randomized controlled trial in Australia conducted by Hersey (personal communication, 2000) showed that both the control group (observation) and the treated group given a viral oncolysate vaccine lived far longer than expected, leading to an insignificant difference between them even though the trend was in favor of the vaccine.

4. MELACINE VS OBSERVATION FOR RESECTED STAGE II INTERMEDIATE THICKNESS (1.5–4.0 MM) MELANOMA: SOUTHWEST ONCOLOGY GROUP TRIAL

The results of this trial, recently analyzed for the first time after 9 yr of study, have been published by Sondak and collaborators (11). A group of 689 patients was randomly assigned to receive Melacine over the course of a year, at the schedule used in our one-arm studies—but unfortunately by the im route—preceded by cyclophosphamide. Patients were required to have no palpable regional lymph nodes, but no formal axillary or inguinal node dissections were required. The sentinel node procedure for determining whether any regional spread had occurred had not yet been developed. With this caveat about the possible heterogeneity of the group as a whole, our assumption is that the inhomogeneity was distributed between the two groups.

The first interesting observation is that the median time to relapse had not been reached in either group with a median follow-up of 8 yr. This was far better than anticipated for the untreated group. When first analyzed in 2000 with a Cox proportional hazards ratio analysis based upon intent-to-treat, the Melacine group had 24% fewer “events” (relapses) than the Observation group with a *p* value of 0.04. When the data were analyzed after another 6 mo, however, the difference had disappeared. By far the more interesting observation was that when patients with certain HLA class I phenotypes were analyzed by Sosman and colleagues, their relapse-free survival was improved considerably. Their response was obscured by their presence in the larger group. This association of HLA phenotype and response is discussed in more detail below.

5. MELACINE FOLLOWED BY INTERFERON- α (IFN- α) IN DISSEMINATED MELANOMA

In 1994 we reported our experience with IFN- α 2B (Intron-A, Schering) following treatment with Melacine in a group of 18 patients (9). This was not a purposeful clinical trial of the combination, but rather an experience with an encouraging response to IFN- α 2B following treatment with Melacine that did not lead to a clinical response, even though immunization was achieved. We noted that 5 million units of IFN- α in that setting led to a major response in eight of them (44%), with site-specific CRs in five.

The median duration of response was 11 mo and the median survival was 36 mo. In contrast, a response rate of 17–20% to IFN- α was anticipated (12). It should be emphasized that, similar to the vaccine itself, the beneficial effects of IFN- α were not seen for at least 2, and usually 4, mo. They were often dramatic at their zenith approx 6–9 mo after initiation of treatment, with clearing of large volumes of disease in the lungs and liver. Patients with liver and bone disease have responded to IFN- α in this setting, which is in direct contrast to our usual experience with Melacine alone (9,13).

By a “landmark” statistical analysis examining only patients who survived at least 4 mo after initiation of IFN- α , which eliminated the biologically poorest patients with early relapse and death, the probability of surviving more than 1 yr was 75% for patients who showed a major clinical response at 4 mo vs only a 13% probability of surviving for more than 1 yr for nonresponders.

Our interpretation of the role of IFN- α was that it may have upregulated HLA class I molecules on the autochthonous tumor in the host, and thus upregulated tumor epitopes presented in those molecules on the cell membrane, permitting T cells that had been elicited by Melacine to recognize their target more effectively. A cytostatic effect of IFN- α on the melanoma independent of the Melacine was less likely, because of the greatly improved response rate relative to IFN- α alone in the wake of the vaccine treatment. A potentiating effect of IFN- α on the activity of T cells was also possible, because that effect has been demonstrated in many previous studies.

5.1. Clinical Trials of Melacine and IFN- α in Combination

To follow up on these encouraging results, we designed several clinical trials of the purposeful combination of Melacine and IFN- α , in various stages of disease.

5.2. Phase II Trial of Combined Melacine and IFN- α in Advanced Disease

The treatment protocol consisted of cyclophosphamide, 300 mg/m² on Day 7, weekly Melacine, 20 million tumor cell equivalents per dose + DETOX adjuvant sc on Weeks 1, 2, 3, 4, and 6, with Intron-A 5 million U/m² sc beginning on the evening of the fourth dose of vaccine and continuing thrice weekly thereafter until relapse. This schedule did not exactly duplicate the one that had produced the results we have just described, but was selected as a compromise in order not to delay IFN- α treatment until after the full course of Melacine had been given. One concern was that the melanoma might progress too far in patients unresponsive to Melacine for salvage by IFN- α . The Melacine was given for an additional course of five injections and then as monthly maintenance if there was no progression of disease at the 8-wk evaluation point.

From October 1994 through October 1995, we registered a total of 56 consecutive patients with documented, measurable metastatic melanoma (stage IV) onto study in this

Table 4
Results of Treatment of Stage IV
Melanoma Patients With Melaccine
and IFN- α

CR	2 (5.3%)
PR	2 (5.3%)
SD or mixed	22 (57.8%)
PD	12 (31.5%)
Total	38 (100%)

CR = complete responses; PR = partial responses; SD = stable disease; mixed = regression of one or more lesions and no change in others; PD = progressive disease

Table 5
Long-Term Responders to Melaccine + IFN- α

Patient	Response	Site	Duration of response	Duration of survival
KDa	CR	Med. LN	>5 yr	>5 yr
CW	CR	Lung	1.8 yr	3.5 yr
AH	PR	Lung	>5 yr	>5 yr
GM	PR	Lung	1.9 yr	2 yr
KV	PR	Lung	3.1 yr	4.3 yr
DG	SD	Lung	2.2 yr	2.3 yr
FJ	SD	Lung	1.2 yr	3.7 yr
RM	SD	SC		>4.9 yr

8 of 38 evaluable patients (21%) (8 of 46 treated patients [17.3%]) lived \geq 2 yr.

CR = complete response; PR = partial response; SD = stable disease; med LN = mediastinal lymph nodes; SC = subcutaneous; surg = surgery; rad = radiation therapy; chemo = chemotherapy; IL-2 = interleukin-2

trial. Ten patients signed a consent form but were then found to be ineligible. Forty-six patients were treated, with 8 of them having rapid relapses before the 8-wk evaluation point. We will present data on the 38 evaluable patients and on all 46 entered (intent-to-treat). Table 4 summarizes these data.

Although the high rate of regression found in our first experience with IFN- α following Melaccine—where the IFN- α was given beginning approx 4 wk after a complete course of Melaccine had been completed—was not reproduced, it was still encouraging that beyond the objective remission rate of 10.6%, with 2 CR and 2 PR, there was a large proportion of patients (57.8%) who were stable at the 4-mo evaluation point and who have continued to survive on maintenance treatment with the regimen. With a median follow-up now exceeding 5 yr, the median duration of a PR or CR is 4 yr. All four patients with a major objective response and four with stable disease exceeding a year have had a survival exceeding 2 yr, including two patients, 1 CR, 1 PR, who have survived more than 58 mo as of February 1, 2000. These responses, their durations, and the resultant overall survival are shown in Table 5. The median survival for the group as a whole was 393 d, or approx 13.2 mo, which exceeded our expectation of 8 mo, even though most of the responses were less than a PR (14).

5.3. Randomized Phase III Study of Melaccine + IFN- α vs High-Dose IFN- α in Resected Stage III Disease

We have nearly completed a phase III randomized, controlled trial of Melaccine + IFN- α in resected stage III melanoma, based upon the information with melanoma lysates alone as well as our experience with Melaccine + IFN- α in stage IV disease. Our multicenter trial at 25 centers throughout the United States compares high-dose IFN- α , given by the same regimen as in ECOG 1684 (20 million units/m² iv for 4 wk, followed by 10 million units/m² three times a week sc for the remainder of a year), with a regimen of Melaccine sc and IFN- α sc at 5 million units/m² three times a week, beginning 4 wk after the first injection of Melaccine. The Melaccine + IFN- α group are given a 2-yr course of treatment, to cover the period of highest risk of recurrence (12–18 mo). Target accrual for this study is 604 patients, 302 in each arm, which is designed to demonstrate a 50% superiority of the lower-dose regimen over high-dose IFN- α , with a power of 90%. We have accrued all 604 patients to the study as of 1/31/03. Both regimens have been well tolerated, particularly the Melaccine + IFN- α arm, where no severe adverse events related to treatment have been reported. Twelve severe adverse events have been reported in the high-dose IFN arm, with 6 probably related to treatment. Discontinuation of DETOX after five injections has minimized local granulomas and abscesses.

We are hopeful that the responses elicited by Melaccine \pm IFN- α with large numbers of tumor cells in disseminated disease—with long durations of survival in patients who have stable disease or greater—will translate into long durations of relapse-free and overall survival in the group of patients with the far more favorable circumstance of minimal residual disease.

6. IMMUNOLOGICAL AND IMMUNOHISTOCHEMICAL OBSERVATIONS

6.1. Immunological Studies

We measured the frequency of precursors of CTLs during immunization with melanoma lysates, and found an association between an increase in frequency and response to treatment (16,17). The work was performed when a full awareness of the importance of HLA class I matching between T cell and tumor was not yet appreciated. However, we were fortunate that, by coincidence, most of our cell lines were HLA-A2 positive, and many others had HLA-A2 supertype alleles (18) permitting us to measure the responses of the majority of our subjects. Thus, only those patients who had an increase in pCTL frequency had a clinical response, whereas those who failed to achieve an increase invariably failed to respond clinically. Approximately 40% of patients with an increase in pCTL frequency had a response (20% clinical responses/50% immunological responses), leaving the question of why all immunological responders did not have a clinical remission. It is likely that a variety of factors, including trafficking of T cells to the tumor, presence of HLA class I and II antigens on the tumor, and production of inhibitory cytokines (such as IL-10 and transforming growth factor [TGF]- β) and yet undetermined influences were responsible for that failure. Nevertheless, the studies showed that T cells were probably involved in the process of rejection, which was reinforced by our histological observations.

Another interesting immunological observation, whose full significance is still uncertain, was that CD4+ T cells with cytotoxic activity were identified in patients treated with

melanoma vaccine, as well as in untreated melanoma patients (19). These CD4+ T cells required longer assays (18 h) to be demonstrated, as well as preincubation of the tumor with IFN- γ . The T cells were HLA class I restricted, as shown in blocking experiments, in obvious contrast to the HLA class II restriction of helper CD4+ T cells. Whether the epitopes recognized were different from those of CD8+ T cells is uncertain. The role of CD4+ cytolytic T cells in rejection is not established, but CD4+ T cells are emerging as very important helper elements for maximal effectiveness of CD8+ cells in adoptive transfer situations.

6.2. Association of Specific HLA Class I Molecules With Response to Melaccine

If cytolytic T cells were important for achieving a clinical response, we speculated that responding patients might have an overrepresentation of certain specific HLA class I molecules known to restrict CTL responses (20). Seventy patients with metastatic melanoma were studied first. Those patients who had HLA-A2, or the closely related HLA-A28 (now called HLA-A68), HLA-B12 “split” (including HLA-B44 and HLA-B45) or HLA-C3 had a far greater likelihood of responding to Melaccine than those who lacked those alleles. Patients with all three alleles had a 40% response rate, whereas those who lacked all three had nearly 0% response. The group as a whole had a 20% response rate.

In a confirmation and extension of these data, Sosman and colleagues (21), analyzing a Southwest Oncology Group study of 689 patients with resected stage II melanoma, found that those with HLA-A2 or HLA-C3 had a longer disease-free survival than those with other HLA phenotypes. Among patients who matched at least two of the five alleles (called “M5”), the vaccine-treated patients had a 5-yr relapse-free survival of 83% vs 59% for those who did not ($p = 0.0002$). The major components of this effect were contributed by HLA-A2 and HLA-C3. Among those who were HLA-A2 positive and/or HLA-C3 positive, the 5-yr relapse-free survival for vaccinated patients was 77%, compared with 64% for observation ($p = 0.004$). There was no impact of HLA-A2 and/or HLA-C3 expression among observation patients. Processed melanoma peptides found in Melaccine may be presented by HLA-A2 and HLA-C3 and play a role in preventing relapse in vaccinated patients.

Although Sosman’s study was not of patients prospectively randomized to treatment by HLA phenotype, the level of statistical significance of the association was striking. The study will therefore be repeated with prospective randomization by HLA class I type. Although heretofore we were unwilling to exclude patients with HLA class I phenotypes other than HLA-A2, -B12, or -C3 from treatment with Melaccine in advanced disease, it may now be prudent to do so, particularly in the adjunctive setting where high-dose IFN- α is an alternative with some degree of proved effectiveness. The confirmatory data of Sosman et al. are sufficiently striking that exclusion of those with the “wrong” phenotypes may no longer be difficult to defend either on ethical or scientific grounds.

6.3. Immunohistological Studies

We also studied the immunohistology of lesions that were undergoing rejection after vaccine therapy in a group of seven patients, compared with a group of six untreated melanoma patients, and other control subjects, including a patient who had a DETOX-induced granuloma at the site of injection, which allowed us to compare the rejection site

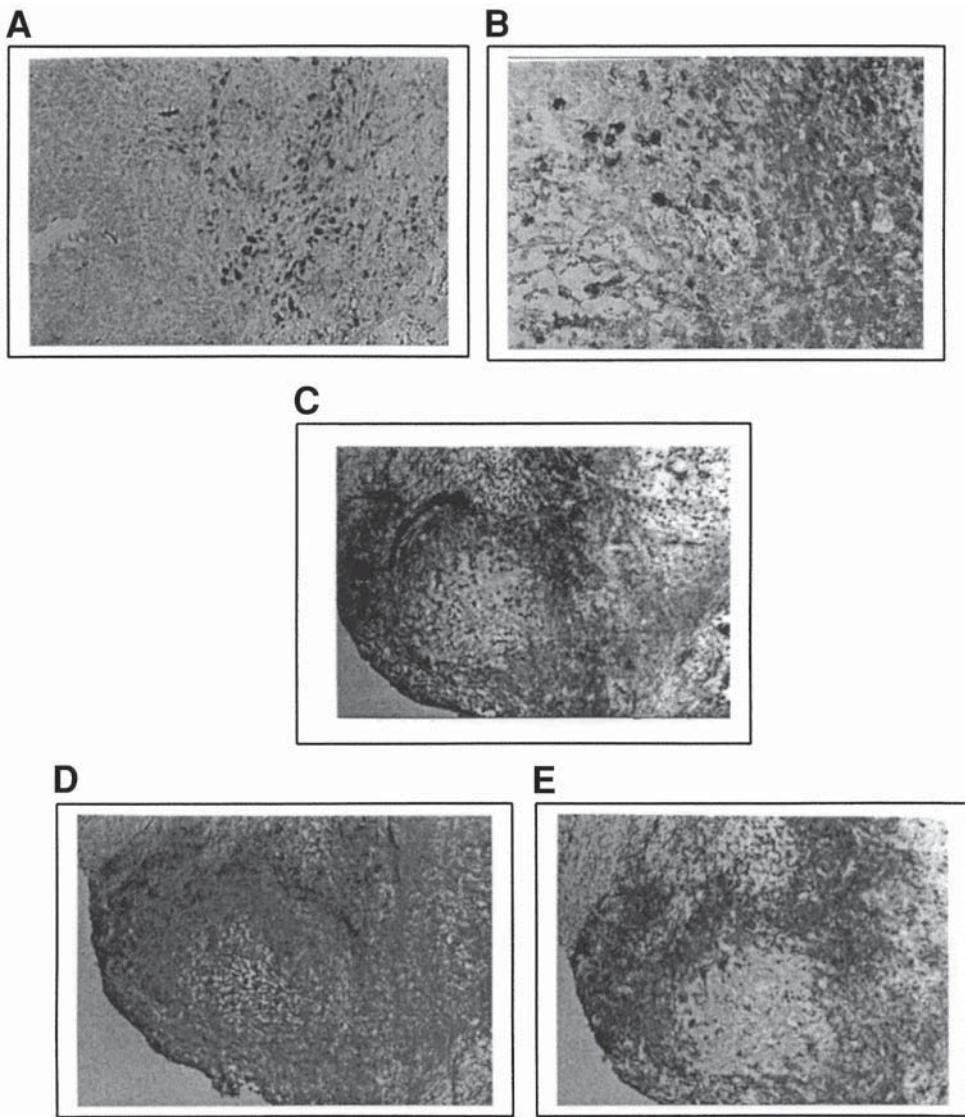


Fig. 3. Immunohistology of a melanoma lesion undergoing rejection after treatment with allogeneic lysates after staining with specific monoclonal antibodies to cluster determinants. (A) CD8+ T cells, (B) CD4+ T cells, (C) Macrophages, (D) HLA class I antigens (w6/32), (E) HLA class II antigens. Note the predominance of CD4+ T cells over CD8+ T cells in the lesion, the peripheral and perivascular distribution of T cells in general, and the predominance of macrophages over all. HLA class I molecules (by beta-2 microglobulin staining) were present in all 11 lesions examined from six patients, whereas HLA class II molecules were absent from the tumor in the same lesions. CD4+ T cells and macrophages were HLA class II positive, however. (Original magnification: A and B $\times 250$; C, D, E $\times 125$.)

with a classical delayed-type hypersensitivity (DTH) reaction (Mitchell MS et al., unpublished data). It was interesting that, contrary to our expectations, the predominant cell in all of the sites of rejection was the monocyte-macrophage, identified with anti-CD11c (Leu M5), anti-class I and II antigens, and usually anti-CD4. CD4+ T cells and CD8+ T cells were present at the periphery of the tumor and perivascularly, with CD4+ cells predominating 3:1 or 3:2 (Fig. 3).

To our pathologist, the lesions most resembled a DTH reaction although the granuloma was far more intense in its representation of T cells and macrophages. HLA class I molecules (identified with monoclonal antibody w6/32 against beta2 microglobulin) were present on the tumor in all specimens undergoing rejection, and in all control melanoma specimens. In this study, performed in the early 1990s, we did not attempt to study the loss of specific HLA class I alleles from the tumor (20,21), and so cannot rule out downregulation of specific molecules even though HLA class I molecules in general were present. In contrast, HLA class II molecules (identified with monoclonal antibody L227) were absent from the tumor in all 11 specimens from seven patients whose lesions were regressing after treatment with melanoma lysates. However, HLA class II molecules were present on the tumor (3+ staining intensity) in specimens from 7 of 11 untreated melanoma patients, and 5 of 6 patients who had received other types of immunotherapy 2–3 mo earlier. Whether the loss of HLA class II molecules makes tumor cells somehow more vulnerable to immunological attack, and their presence confers resistance, or alternatively whether class II molecules are lost as a consequence of immunological attack are interesting open questions. In favor of the first set of premises, the inverse correlation of HLA class II expression on melanoma and sensitivity to immunotherapy is consistent with the findings of Ferrone and coworkers, who emphasized that primary melanomas that expressed HLA class II molecules had a poorer prognosis than those with absent HLA class II (22).

Despite the association of clinical response with rises in precursors of CTLs, a direct cytotoxic role of either CD4+ or CD8+ T cells was not supported by these observations. Rather, a more indirect role is suggested, perhaps involving cytokines or chemokines released by the T cells.

6.4. Schema of Immune Response to Allogeneic Lysates

We suggest that allogeneic lysates are taken up by dendritic antigen-presenting cells in the dermis after injection, and then processed and presented in self-HLA-class I and -class II context to CD8+ and CD4+ T cells, respectively. The addition of alloantigens, far from preempting the immune response to weaker tumor-associated antigens, may well augment the response, by eliciting additional T-cell help, in the form of cytokines such as IL-2, from alloreactive T helper cells. The additional cytokines from the alloreactive T cells may augment CD4+ and CD8+ T-cell activity against the tumor, in some cases leading to its destruction if all other elements enabling response are present, such as adequate representation of HLA molecules and tumor epitopes on the tumor cells, and lack of antagonism of T cells by cytokines released from or elicited by the tumor. This schema, which is illustrated in Fig. 4, was presented previously in a similar form after our data from early trials were first analyzed (23).

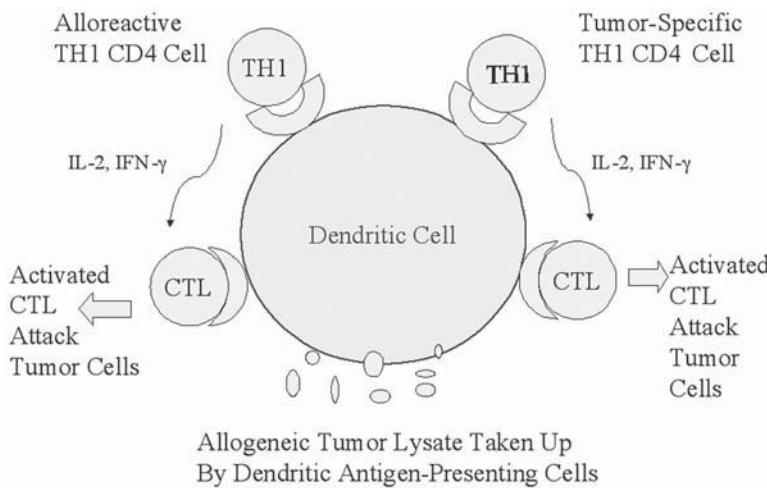


Fig. 4. Schema of the mechanism by which allogeneic lysate vaccines may immunize against a tumor. Uptake and processing of the lysates by dendritic cells after injection may lead to the production of CD4+ and CD8+ T cells directed against the tumor. Presentation of exogenous epitopes by HLA class II molecules to CD4+ cells and by HLA class I molecules to CD8+ T cells (cross-priming) have both been demonstrated. Alloantigens may augment T-cell help to CD4+ and CD8+ T cells, through stimulation of alloreactive T cells to produce cytokines, in addition to those produced by tumor-specific T cells immunized by tumor antigens. Thus, alloimmunization may be beneficial to immunity directed at the tumor.

7. OTHER CLINICAL TRIALS WITH ALLOGENEIC LYSATES

Other investigators have also concluded that allogeneic materials are potentially of greater benefit to patients than autologous tumor-derived immunogens. Among the earliest of these studies were those of Cassel and associates, who utilized viral oncolysates of melanoma cell lines as immunogens (24). These studies were extremely promising, with a number of remissions lasting a decade or more, but unfortunately may have been ahead of their time. Attempts to reproduce the results in large-scale cooperative trials were unsuccessful, but those were hampered by failure of attention to important details, such as viability of the cells before viral treatment.

Wallack immunized patients with allogeneic viral oncolysates created with vaccinia virus, and did one of the first double-blind, randomized, multicenter studies in 250 patients—perhaps too small a number to show a small difference between the groups—with resected stage III melanoma. Protein representing 5 million melanoma cells—in retrospect perhaps too low a dose by at least fourfold—was injected with $10^{5.6}$ 50% tissue culture infectious dose of vaccinia virus. There was no difference between the immunized group and observation, although subset analysis suggested there was a subgroup that may have benefited (25). Such analyses are fraught with danger, because a proportion of the subsets may be “significant” statistically simply because of our acceptance of a 0.05 level of type 1 error (where there are 5 chances in 100 of finding a “significant” difference that may in fact be insignificant). Nevertheless, the study was an important precedent for studies with vaccines, which require large numbers of patients treated at several institutions and a long-term follow-up to demonstrate definitively a difference from a control

population. Morton and colleagues have also extensively studied an irradiated melanoma vaccine, (CancerVax) given with BCG, and describing prolonged survivals of early stage resected patients in Phase II trials (26).

A preliminary study involving an interesting construct of melanoma lysates adherent to microscopic (5 μm) amorphous silica beads, called large multivalent immunogen or LMI, should be mentioned. In this work, fifteen patients were treated with one of three levels (10, 20, or 100 million tumor cell equivalents) for three monthly doses and had no toxicity. An increase in CTL precursors of 2- to 10-fold was detected in 9 of the 15 patients (27). Five patients had stable disease at 3 mo continued treatment. Clinical regression of disease was noted in a pulmonary nodule in two patients, one of whom had a *bona fide* PR. Further studies with similar LMI preparations in other diseases are in progress (Okazaki I, Miller J, and Mescher M, personal communication, 2003).

8. CONCLUSIONS AND FUTURE

Our personal experience with allogeneic melanoma lysate vaccines, given to several hundred patients, has convinced us that although the response rates in disseminated disease are at most in the range of 15–20%, there is indeed activity of these preparations even when billions of tumor cells are present. There have been a number of patients with a gratifying dramatic increase in survival lasting 5–10 yr or more. Allogeneic lysates may perhaps be effective in preventing recurrence of melanoma in patients with resected stage III disease, and have been equivalent to toxic four-drug chemotherapy even when given by a suboptimal route of administration in a large multicenter trial. With the same caveat, suboptimal (im) route of administration, a large Southwest Oncology Group phase II study of Melaccine vs observation in resected intermediate thickness stage II melanoma was initially positive at a follow-up of 1 yr ($p = 0.04$), although further follow-up showed no difference. Melaccine was approved for use in Canada in November 1999, based primarily upon the data in disseminated melanoma that we have reviewed here, and particularly upon its safety and maintenance of a good quality of life. Regardless of whether Melaccine or another allogeneic vaccine is eventually approved in the United States, we will continue to pursue multiantigenic vaccines as a treatment for the various stages of melanoma. More defined vaccines are now possible because of an expansion of knowledge of which antigens are shared among melanomas. Preselection of responders with HLA class I haplotyping also appears feasible.

In the future, vaccines made with a predetermined mixture of peptides, proteins, or the DNA encoding them, may well supplement or supplant lysate vaccines such as Melaccine, but for the immediate future the use of lysates injected directly sc into patients or incorporated into dendritic cells injected sc and/or id appears to be a useful approach to melanoma treatment. Combinations of vaccines and cytokines, and vaccines with adoptively transferred *in vitro*-immunized T cells, are also very promising. However, it is becoming all too clear that the addition of agents that inhibit the tumor's ability to antagonize the immune response will probably be essential in order for any immunotherapy to be optimally effective, even at early stages in the progression of the tumor.

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CONTENTS

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1. INTRODUCTION

It is estimated that approx 30,000 new cases of renal cell carcinoma (RCC) were diagnosed in 2002 (1). Approximately 20–30% of patients with RCC present with metastatic disease and their overall median survival is 6–8 mo (2). Although, metastatic RCC carries a poor prognosis, in a small subset of patients the disease has a variable course (3). Oliver et al. observed 73 patients with metastatic RCC without treatment (4). In this series, 4% had complete and spontaneous regression of disease and 3% had partial regression of disease. Furthermore, 5% had stable disease with no evidence of progression for more than 12 mo. There are also numerous cases of patients who recur with metastatic RCC over 10 yr after undergoing a nephrectomy for apparently localized disease. It is presumed from these observations that the immune system plays a key role in cases of spontaneous regression and durable remissions of RCC.

Several observations underscore the importance of the immune system in determining the course of RCC. Metastatic RCC is sensitive to generalized stimulation of the immune system with cytokines. The systemic administration of interleukin-2 (IL-2), either alone or in combination with interferon- α , can produce objective response rates ranging from approx 15 to 20% (5–7). Further evidence for the role of the immune system comes from

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Table 1
Tumor Antigens Expressed by RCC

Antigen	Reference
G250	32
EGFR	11–13
KIT (in chromophobe)	54
thymidine phosphorylase	55

multiple reports of a direct relationship between a favorable response to immunotherapy and the development of autoimmune thyroiditis (8). The development of autoimmunity may be the byproduct of successful immunotherapy.

RCC also has several necessary components required for a successful immune response. First, although few have been described thus far, tumor antigens do exist on RCC (Table 1). G250, also known as CA IX and MN, is present in over 80% of primary and metastatic tumors, and it is present in 95–100% of the clear-cell variant, which is the most common histologic type (9,10). In RCC, the overexpression of G250 is the direct consequence of a mutation in the von Hippel-Lindau (VHL) gene, which normally functions to suppress G250. Epidermal growth factor receptor (EGFr) is also expressed by RCC (11–13). Monoclonal antibodies with the ability to bind to G250 (14,15) and EGFr (16) have entered clinical trials and appear able to localize to RCC. Second, RCC can be destroyed by cellular immune effectors such as lymphokine-activated killer (LAK) cells, tumor-infiltrating lymphocytes (TILs), and activated memory lymphocytes. Although it is not the purpose of this chapter to discuss adoptive cellular immunotherapy, clinical activity has been observed with these approaches (reviewed in ref. 17). These studies suggest that RCC can be recognized *in vivo* in both an major histocompatibility complex (MHC)-restricted and -nonrestricted fashion and that effectors can traffic to RCC. Therefore, it is hoped that effectors induced by vaccines will have similar capacities.

2. VACCINES TARGETING RCC

2.1. Dendritic Cell-Based Therapies

A possible role of antigen-presenting cells (APCs) such as dendritic cells (DCs) in immune responses to RCC is supported by the observation that patients receiving interferon- α for metastatic RCC had a better prognosis if they had high levels of DCs in their nephrectomy specimen (18). Furthermore, Thurnher identified DC populations migrating from human RCC tumor explants (19). These cells displayed the characteristics of mature DCs including: expression of high levels of MHC and costimulatory molecules (CD88, B7-2), ability to stimulate naïve T cells in a mixed-leukocyte reaction, and a reduced potential to capture soluble Ag. Up to 9% of these emigrating leukocytes expressed CD83, a specific mature DC marker. These CD83+ cells were 40-fold enriched in tumor as compared to peripheral blood lymphocytes (PBLs). Others have found fewer DC-infiltrating tumors (20). In either case, there is concern that the tumor-associated DCs may be suppressed by tumor-related factors. Therefore, it has been hypothesized that

generation and ex vivo loading of DCs for use as vaccines would have greater efficacy by eliminating any negative influences from the tumor microenvironment.

2.1.1. DC GENERATION FROM PBLs IN RCC PATIENTS

The development of in vitro DC culture methods has allowed for their study, and burgeoning clinical use. The UCLA Kidney Cancer Program has performed in vitro studies characterizing DCs obtained specifically from patients with RCC (21). Using common cytokine-based culture techniques (granulocyte-macrophage colony-stimulating factor [GM-CSF], IL-4 plus 10% autologous serum), DCs can be consistently isolated and expanded from the PBLs of patients with mRCC. When loaded with autologous RCC TuLy and cocultured with autologous TILs, these DCs induce (a) lymphocyte growth expansion; (b) upregulation of CD3+, CD56+ cell populations; (c) enhancement of autologous tumor lysis; and upregulation of TILs, Th1 cytokine profile. Other studies confirm these findings including the ability to consistently generate functional DCs from patients with RCC (22).

These in vitro DC culture methods require considerable resources when considering clinical/therapeutic applications including: large blood draws or leukapheresis, isolation of PBLs, culture/differentiation in sterile conditions, loading with Ag and patient vaccination. At UCLA, we have also demonstrated via a novel phase I dose-escalation trial, that DCs can be generated in vivo by administering GM-CSF plus IL-4 subcutaneously to patients with advanced cancers (including subjects with RCC) (23,24). These patients show minimally detectable DCs at baseline, and have a marked increase in functional circulating DCs after 7–14 d of combined cytokine administration. These observations will underlie future DC-based clinical trials without the need for ex vivo processing of patient PBLs to generate DCs.

In addition, GM-CSF and IL-4 may have a primary therapeutic effect for metastatic RCC (23). In a recently completed phase I trial, 14 patients with metastatic RCC were treated with GM-CSF and IL-4. Although no objective responses were noted, one patient had stable disease for 24 mo and another patient with widely metastatic disease had stable disease for over 40 mo.

2.1.2. DENDRITIC CELL-BASED CLINICAL TRIALS

At UCLA, phase I clinical trial for patients with mRCC, using DCs pulsed with autologous TuLy has been completed (25). Cytokine-derived DCs were obtained from peripheral blood precursors cultured in GM-CSF and IL-4, and lysates were obtained from primary nephrectomy specimens. We have consistently prepared this vaccine for three consecutive weekly intradermal vaccine applications. No dose-limiting toxicities were observed. One subject showed in vitro evidence of enhanced antitumor immunity with upregulation of Th-1 cytokine production from PBLs and enhanced cytotoxicity against autologous tumor. This subject sustained a short-lived partial response, then developed brain metastases.

In a similar study, 10 patients with metastatic RCC were treated with DCs (26). The DCs were pulsed with tumor lysate when patients underwent a nephrectomy and tumor tissue was available. In this early-phase study, treatment resulted in immune stimulation as measured by increase in peripheral immune cells, and one partial response was noted. A study from Austria used cytokine-generated DCs loaded with TuLy plus keyhole limpet hemocyanin (KLH) for intravenous application (27). Again the vaccine was well

tolerated, and in this study, there was evidence of in vitro immune response to KLH and TuLy. Furthermore, 2 of 27 patients had a complete response and 1 patient had a partial response.

In a mouse model it was found that IL-2 could augment the efficacy of a TuLy-pulsed DC vaccine (28). This concept was applied in a phase I study where 12 patients with metastatic RCC were treated with TuLy-pulsed DCs and low-dose IL-2 (29). No objective responses were seen, and no cellular or humoral response to the TuLy was found after treatment, suggesting that this vaccine strategy is ineffective. At this time the effectiveness of DC vaccines loaded with TuLy remains to be defined in larger phase II trials.

Recently a tumor cell–DC hybrid vaccination has demonstrated remarkable clinical response in patients with RCC (30). To prepare this vaccine, allogeneic cytokine-derived DCs plus autologous tumor cells are hybridized using an “electrofusion” process. Allogeneic DCs are used to recruit allo-reactive helper T cells. Seventeen subjects were vaccinated subcutaneously with a booster vaccine given after 6 wk. All subjects without disease progression received further booster vaccines every 3 mo. Interestingly, a different allogeneic DC donor was used to prepare each vaccine. There were four complete and two partial responses observed (mean follow-up time of 13 mo), with mild to moderate toxicity (fevers, tumor pain) and no evidence of autoimmune reaction. All subjects with objective response demonstrated a positive delayed-type hypersensitivity reaction to autologous tumor. Although this study is often cited, the study has been shrouded in controversy. The researchers were criticized for lack of diligence in carrying out the clinical study, however, no evidence of scientific misconduct was found (31). A phase I/II trial of RCC–DC hybrid sponsored by Genzyme Corporation is currently being conducted.

DCs loaded with tumor-derived genetic material is another way to expose DC to the entire complement of tumor-expressed antigens. Vieweg and colleagues have demonstrated that autologous dendritic cells transfected with total renal tumor RNA are potent stimulators of CTLs and antitumor immunity in vitro. In a Phase I trial of this approach, 10 patients with metastatic RCC received renal tumor RNA-transfected dendritic cells. No vaccine-related adverse effects were observed. In six of seven evaluable subjects, expansion of T cells specific for a variety of tumor antigens such as telomerase reverse transcriptase, G250, and oncofetal antigen was detected after immunization.

3. TUMOR-ASSOCIATED ANTIGEN VACCINES

The demonstration that some RCC lines express antigenic determinants that can be recognized by MHC-restricted cytotoxic T lymphocytes (CTLs) has led to efforts aimed at identifying tumor-associated antigen (TAA) in human RCC. One recently cloned candidate RCC-TAA is G250 (32). DCs loaded with G250 peptide and cultured with autologous T cells can be used to generate human CTLs capable of lysing G250-expressing targets (33). This suggests that TAA-based immunotherapeutic strategies may be effective for RCC patients. In an effort to further enhance the immunogenic potential of G250, a fusion protein combining G250 and GM-CSF as been engineered at UCLA (34). This construct has been shown to effective as a RCC vaccine in a murine model and a clinical trial for metastatic RCC is being planned.

Heat shock protein (HSP) is another TAA that is being evaluated for the treatment of metastatic RCC. A phase I clinical trial of an HSP vaccine for RCC has demonstrated objective responses, prolonged disease stabilization (35), and stable or improved quality

of life during treatment (36). A pivotal, 500-subject phase III trial of adjuvant HSP vaccine in high-risk, nonmetastatic RCC is under way.

4. GENE THERAPY-BASED APPROACHES

4.1. *Gene-Modified Tumors*

Tumor cells transfected with genes for IL-2, IL-12, and GM-CSF, or MHC or costimulatory molecules represent a platform for increasing the immunogenicity of the tumor cells. A phase I human gene therapy trial using cultured, irradiated autologous RCC cells either unmodified or genetically modified ex vivo to secrete GM-CSF in patients with metastatic RCC has been reported (37). No significant toxicities were seen. Biopsies of the intradermal site of vaccine injection revealed APCs and T-cell infiltrates in addition to eosinophils and neutrophils. One partial response was observed in a patient who also had the largest delayed-type hypersensitivity conversion to unpassaged autologous tumor. Another group in Japan has made attempts to improve upon this vaccine strategy by using partially human leukocyte antigen (HLA)-matched allogeneic tumor cells (38).

Investigators at Memorial Sloan Kettering Cancer Center have treated 12 patients with RCC with a tumor vaccine consisting of an irradiated HLA-A2+ allogeneic RCC cell line transfected with the IL-2 gene (39). Only Grade I and II toxicities were observed, but there were also no objective responses. Pertaining to safety, there was no detection of the helper virus in any patient 1 wk after vaccination. Another strategy using B7-1 gene-modified autologous tumor cells has been used in combination with systemic IL-2 in a phase I trial (40). As anticipated, the toxicity was related to the IL-2.

4.2. *Intratumoral Gene Therapy*

Technology using liposomes to transfer genes into cells without the need for viral vectors has evolved into clinical trials in RCC. One trial involves the direct injection of a lipid-formulated plasmid DNA encoding for the foreign class I MHC antigen, HLA-B7 “Allovectin-7” (Vical Inc., San Diego, CA) into accessible metastatic RCC lesions (41). Fifteen HLA-B7-negative patients were treated, without significant toxicity or objective responses. Detection of HLA-B7, mRNA, and protein expression could be demonstrated in 8 of 14 tumor samples. A similar trial using direct gene transfer of the IL-2/liposome complex “Leuvectin” (Vical Inc., San Diego, CA) into accessible metastatic RCC lesions indicate that Leuvectin is safe, is free of systemic toxicity, and has biologic activity (42). Further phase II trials in RCC are planned (43).

5. OTHER USES OF RCC VACCINES

5.1. *Vaccine-Primed Lymph Node Cells*

Lymph nodes that drain malignancies or sites of tumor vaccination presumably harbor preeffector cells that can be activated and expanded ex vivo using anti-CD3 antibody and low concentrations of IL-2. In an early-phase clinical trial, 12 patients with metastatic RCC were treated with vaccine-primed lymph node (VPLN) cells and high-dose bolus IL-2 (44). There were two complete and two partial responses. In a similar study, 20 patients with metastatic RCC were treated. The therapy was well tolerated and there was one partial response.

These feasibility studies prompted the initiation of a phase II trial using VPLN cells plus IL-2 for RCC (45). Vaccines consisted of autologous tumor cells irradiated and admixed with Tice bacille Calmette-Guérin (BCG). Seven days after vaccine administration, the VPLN cells were harvested from the draining lymph nodes and activated using anti-CD3 and IL-2. These cells were then adoptively transferred with high-dose bolus IL-2 (360,000 IU/kg). Of 34 patients available for evaluation there was a 27% response rate.

In the hopes of improving response rates, various modifications to the treatment protocol have been proposed. The lymph nodes can be primed with DCs loaded with tumor lysate (46). In a recent publication using a murine model, potent tumor-reactive effector cells were generated by doubly activating the VPLN cells using both anti-CD3 and anti-CD28 (costimulatory signal) (47).

Gene-modified tumors have also been used as the lymph node-activating vaccine. For example, investigators primed subjects with autologous tumor cells modified ex vivo to express HLA-B7 and β 2-microglobulin (48). Lymphocytes from lymph nodes draining the vaccination site were obtained and expanded ex vivo with anti-CD3 and IL-2, and adoptively transferred to 9 patients with melanoma and 11 patients with metastatic RCC. In this phase I/II study no objective responses were observed.

5.2. *In Vitro Immunizations With Gene-Modified Tumors*

Alternatively, gene therapy techniques can be used in the preparation of TILs for adoptive immunotherapy. Investigators at UCLA have propagated TILs in vitro in the presence of a tumor cell line infected with the IL-2 gene (RCC-Ad-IL-2) (49). Compared to standard TIL growth conditions in exogenous IL-2 alone, TILs grown in the presence of RCC-Ad-IL-2 were more immunologically potent.

6. CONCLUDING REMARKS

Vaccine-based therapies for RCC are early in development, in part because of the relative paucity of identified tumor antigens. Also, most of the clinical trials have been very small and therefore, difficult to interpret (50). Nonetheless, the sensitivity of RCC to immune effectors supports the continued enthusiasm for these approaches. Recent promising results for nonmyeloablative allogeneic stem-cell transplantation for RCC also raise the possibility that vaccines will be administered in the post-transplant setting and augment the observed activity further. Also, because there is growing evidence implicating T-cell dysfunction associated with RCC (51–53), we predict that future efforts will focus on both activating the immune system as well as overcoming tumor-related immunosuppression.

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Vaccines for Hematological Malignancies

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CONTENTS

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1. INTRODUCTION

High-dose chemotherapy supported by autologous stem-cell transplantation has shown considerable promise as an effective and, in some situations, potentially curable approach to hematological malignancies including leukemias, lymphomas, and multiple myeloma (MM), which are otherwise refractory to conventional chemotherapy modalities. This is attested to by the substantial increase in the number of complete remissions experienced by patients over what had been achieved with standard therapy, and by the significant prolongation of event-free and overall survival times (1). However, despite advances in supportive care, continuing refinement of chemotherapy-conditioning regimens, and combinational maintenance therapy, relapse of the underlying diseases remains the primary cause of treatment failure (1). Thus, novel therapeutic approaches that have a mode of action different from and non-cross-resistant with cytotoxic chemotherapy are required to eradicate tumor cells that have become multidrug-resistant. For this reason, strategies for post-transplantation immunomodulation would be desirable. To this end, immunotherapy aimed at inducing or enhancing tumor-specific immunity in tumor-bearing patients may be useful for the control or even eradication of the remaining tumor cells.

Immunotherapy has now become an important part of therapeutic strategies for hematological malignancies. Passive immunotherapies using monoclonal antibodies directed against tumor-associated surface antigens, such as CD20 (rituximab; Rituxan), CD22 (epratuzumab; LymphoCide), CD52 (alemtuzumab; Campath), and major histocompatibility complex (MHC) class II (Hu1D10; Remitogen), have been extensively studied

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either alone or in combination with chemotherapy or with other biological agents (for reviews, *see* refs. 2–4). These reagents can be applied as conjugates with toxins or isotopes as means to deliver a toxic compound or radioactivity to tumor cells, or as unlabeled antibodies to cause direct anticancer effects or induce a secondary immune response against tumor cells via a number of mechanisms. Thus far, encouraging results have been obtained in the treatment of various hematological malignancies, including non-Hodgkin's lymphomas, chronic lymphocytic leukemia, Waldenström's macroglobulinemia, and MM (2–4). Active immunotherapy, in which the patients are induced to generate a specific immune response against the tumor cells, has long been a goal of tumor immunologists. Although no active immunotherapy maneuver has yet proven to be effective in the clinic, intensive efforts are under way to develop such an approach. Experiments in animal models have shown that vaccination against actively growing tumors is much more difficult to accomplish (5,6). It is therefore not surprising that clinical trials in patients with gross disease will be the most difficult setting in which to demonstrate efficacy. Thus, it is conceivable that immunotherapy may work better in patients in remission or with minimal residual disease, who are more likely to be able to generate a robust immune response against the tumor and to derive therapeutic benefit.

Recent insights into immune system function have fostered a better appreciation of the role of specialized antigen-presenting cells (APCs) termed dendritic cells (DCs). Initially described by Steinman and Cohn (7) in 1973 as an adherent cell type among mouse splenocytes with a distinctive stellate morphology, DCs soon became known for their role as the sentinels of the immune system (for reviews, *see* refs. 8 and 9). In their immature state, DCs reside in peripheral tissues, where they survey for incoming pathogens. An encounter with pathogens leads to DC activation and migration to secondary lymphoid organs, where they trigger a specific T-cell response. DCs are cells that not only can stimulate quiescent naïve CD4⁺ and CD8⁺ T cells and B cells and initiate primary immune responses, but can also induce a strong secondary immune response at relatively low numbers and with low levels of antigen. Furthermore, DCs are involved in polarization of T-cell response via secreted cytokines and in induction of tolerance through deletion of self-reactive thymocytes and anergy of mature T cells (8,9). Given their central role in controlling immunity, DCs are logical targets for many clinical situations that involve T cells, such as transplantation, allergy, autoimmune disease, resistance to infection and to tumors, immunodeficiency, and vaccination. Consequently, trials of DC vaccination for a variety of human cancers have been completed or are under way. This chapter discusses and summarizes vaccination studies, including DC-based vaccines, in hematological malignancies. As several other chapters are devoted to the development of preclinical vaccination strategies, clinical experience of tumor vaccine approaches for the treatment of hematological malignancies is the focus of this chapter.

2. VACCINES FOR LYMPHOMAS

2.1. *Idiotype-Based Protein Vaccines for Follicular B-Cell Lymphoma*

Idiotype (Id) structures present on secreted monoclonal immunoglobulin (Ig) and on the surface Ig of clonal B cells in B-cell malignancies are tumor-specific antigens; as such, they are potential targets for specific anti-Id immunity (10,11). An intervention aimed at expanding Id-specific T cells with cytotoxic or suppressive effects on the tumor B-cell clone may be a feasible immunotherapeutic approach. Active immunization against

idiotypic determinants on malignant B cells has produced resistance to tumor growth in transplantable murine B-cell lymphoma and plasmacytoma (12–15).

Kwak, Levy, and coworkers at Stanford University pioneered the vaccination of patients with B-cell lymphoma with Id protein derived from the patients' tumors (16,17). In their first report of nine treated patients with low-grade follicular lymphoma primarily at first remission after chemotherapy, each patient received a series of subcutaneous injections of autologous Id protein that had been conjugated to an immunogenic carrier protein, keyhole limpet hemocyanin (KLH). Id-specific humoral and/or cellular immune responses developed in seven of nine immunized patients. Tumor regression was observed in two patients who had measurable disease (16). In these patients, it was subsequently shown that cell-mediated cytolytic immune responses might be an important determinant of vaccine efficacy (18). In their follow-up study, 41 patients received a series of injections with vaccines consisting of Id protein coupled to KLH and emulsified in an immunological adjuvant, either incomplete adjuvant (5% squalane, 2.5% Pluronic L121, 0.2% Tween-80, and phosphate-buffered saline) or complete adjuvant (incomplete adjuvant containing increasing doses of threonyl-muramyl dipeptide). Among the first 32 patients in that trial vaccinated while in first remission, about one-half (14/32) mounted anti-Id immune responses to the vaccine. Long-term follow-up of these 32 patients, compared with nonresponders, revealed that the development of an immune response was strongly correlated with prolonged freedom from disease progression; overall survival was also superior in responding patients (17). These studies clearly demonstrate that Id protein can be formulated into an immunogenic, tumor-specific antigen in humans with lymphoma.

Since publication of the above studies, many groups have reported their results on Id-KLH vaccination in B-cell lymphoma. Among them, a study from Kwak's group at the National Cancer Institute showed a striking clinical benefit of the vaccine for patients (19). The investigators vaccinated 20 patients with follicular lymphoma in first complete remission following chemotherapy with Id-KLH and granulocyte-macrophage colony-stimulating factor (GM-CSF) administered locally as adjuvant. After vaccination, tumor-specific cytotoxic CD8+ and CD4+ T cells were found in 19 of 20 patients. Although anti-Id antibodies were detected, they appeared not to be required for antitumor clinical response. Presence of bcl-2 proto-oncogene product of the t(14;18) translocation characteristic of follicular lymphoma was monitored in the blood of these patients, and clearance of bcl-2 polymerase chain reaction (PCR) signal was achieved in 8 of 11 evaluable patients. In line with these results, a recent study from a Spanish group also showed clinical activity of Id-KLH vaccine in patients (20). Collectively, these studies demonstrate that Id-KLH vaccination is associated with clearance of residual tumor cells from blood and long-term disease-free survival in low-grade follicular lymphoma. Prospective, randomized trials have now begun to seek evidence of clinical benefit after Id vaccination in B-cell lymphoma. In addition, studies are under way to evaluate the efficacy of Id-KLH vaccines to treat patients who have relapsed indolent non-Hodgkin's lymphoma (21) or have undergone myeloablative therapy (22), and patients with mantle cell lymphoma (23–25).

To apply Id-based vaccination to large numbers of patients, it is crucial to develop feasible and rapid methods, other than the currently used hybridoma technique, to prepare Id protein or its fragment from each patient. Several alternative sources of recombinant Id proteins are now available for clinical study. These include Id proteins produced in a

variety of genetically engineered organisms. The first among these that are currently being evaluated under phase I/II clinical trials are Id proteins produced in transfected mammalian cells grown in tissue culture (26) and plant-derived single-chain variable region (scFv) Id fragments (27). It has been shown that Id proteins from lymphoma patients' specimens can be produced in recombinant bacteria and that DCs pulsed with these proteins (secreted as Fab fragments) can stimulate Id-specific cytotoxic T lymphocytes (CTLs) *in vitro* (28). Furthermore, the tobacco mosaic virus has been exploited as a vector for engineering protein production in tobacco plants. In a preclinical study, vaccination of mice with plant-derived Id scFv was able to elicit tumor protection equivalent to that of Id-KLH plus adjuvant (29). On the basis of these results, a phase I/II clinical study has been initiated to evaluate the efficacy of plant-derived Id scFv vaccine in patients with follicular lymphoma (27).

2.2. Dendritic Cell-Based Vaccines for B-Cell Lymphoma

DCs are the most potent APCs and are ideally suited to serve as natural adjuvants for purposes of vaccination and immunotherapy for cancers (30,31). Methods have been developed to obtain substantial numbers of DCs from proliferating CD34+ progenitors in bone marrow and peripheral blood, as well as from nonproliferating precursor cells, such as CD14+ monocytes, in human blood (32–34). Induction of antitumor immune responses by injection of antigen-loaded DCs has been extensively studied in animals. First, it was found that injection of DC-enriched preparations pulsed with tumor lysates (35,36) could protect naïve animals from subsequent lethal tumor challenge. Next, it was shown that administration of density gradient-purified splenic DCs pulsed either with soluble tumor protein antigen expressed by a B-cell lymphoma (37) or with synthetic MHC class I-restricted peptides derived from tumor antigens (38,39) induced protective antitumor immunity. Thus, it was evident that *ex vivo* delivery of purified tumor antigen to DCs could result in an effective tumor vaccine.

Hsu and coworkers (40) were the first to report that DCs pulsed with a tumor antigen could elicit specific tumor-reactive T cells and had clinical efficacy in hematological malignancies. In their pilot study, four patients with low-grade B-cell lymphoma who had measurable disease were immunized with Id-pulsed DCs isolated from peripheral blood. Each patient received three monthly intravenous DC infusions, with a median of 5×10^6 DCs per infusion, followed 2 wk later by subcutaneous booster injections of Id protein and KLH, with a final DC infusion given 5–6 mo later. Measurable antitumor cellular immune responses developed in all patients, and clinical responses were observed in three of the four patients. After this study, 35 more patients were recruited and treated by the same approach (41). Among 10 initial patients with measurable disease, 8 mounted T-cell proliferative anti-Id responses, and 4 had clinical responses (2 complete remissions, 1 partial response, and 1 molecular response). In the subsequent 25 patients, 15 of 23 who completed the vaccination schedule mounted T-cell or humoral anti-Id responses. Among 18 patients with residual tumor at the time of vaccination, 4 had tumor regression, and 16 of 23 patients remained without tumor progression at a median of 43 mo after chemotherapy. Six patients with disease progression after primary DC vaccination received booster injections of Id-KLH protein, and tumor regression was observed in three of them (two complete remissions and one partial remission). Thus, these studies

indicate that Id-pulsed DC vaccination can induce T-cell and humoral anti-Id immune responses and durable tumor regression in B-cell lymphoma.

2.3. DNA Vaccines for B- and T-Cell Lymphomas

In addition to active immunization with purified Id proteins or Id-pulsed DCs, DNA vaccines containing genes encoding Id epitopes and carrier protein may be a convenient alternative vaccine delivery system because Id DNA can be rapidly isolated by PCR techniques. A study by King and coworkers (42) described the use of DNA vaccines with scFv fused to fragment C of tetanus toxin in murine lymphoma and myeloma. The vaccines promoted an anti-Id response and induced strong protection against B-cell lymphoma, which was likely antibody-mediated. The same fusion design also induced protective immunity against a surface Ig-negative myeloma. The same group also prepared and used DNA vaccines encoding a fusion protein consisting of a single-chain T-cell receptor sequence and a pathogen-derived sequence from tetanus toxin and showed that the DNA vaccine was effective in inducing antyclonotypic immunity and protecting against T-cell lymphoma in a mouse model (43). Furthermore, genetic vaccination with Id-encoding recombinant adenovirus (44) or plasmid DNA (45) has also shown efficacy in several murine lymphoma models. On the basis of these results, a phase I/II clinical trial was conducted to examine the safety and immunogenicity of a plasmid DNA vaccine encoding chimeric Id (mouse Ig Fc fragments and a patient's Id determinants) in patients with follicular B-cell lymphoma (46). The first 12 patients received three monthly intramuscular injections of the DNA in three dose-escalation cohorts of four patients each (200, 600, and 1800 µg). After vaccination, 7 of 12 patients mounted either humoral or T-cell-proliferative responses to the mouse Ig, and only one patient had a T-cell response to the autologous Id. In the next 12 patients, to whom 1800 µg of DNA vaccine was delivered both intramuscularly and intradermally with a needle-free injection device, 9 had humoral and/or T-cell responses to the mouse Ig, and 6 exhibited humoral and/or T-cell anti-Id responses. Subsequently, a third series of vaccinations was carried out with 500 µg of human GM-CSF DNA mixed with 1800 µg of Id DNA, and no significant changes or improvement were observed, as compared to the second series of the study. Nevertheless, the vaccination approach appeared safe because no significant side effects or toxicities were observed. Additional studies are needed to optimize vaccine dose, routes of administration, vector designs, and prime-booster strategies in order to improve the efficacy of the treatment.

3. VACCINES FOR MULTIPLE MYELOMA

3.1. Myeloma Cells and Tumor Antigens

MM, characterized by the clonal expansion of malignant plasma cells, is a fatal disease. It constitutes 10% of hematological malignancies in the United States and is more prevalent than lymphocytic leukemia, myelocytic leukemia, or Hodgkin's disease. Myeloma B lymphocytes are mature B cells. They may express, on the cell surface, Id Ig as well as MHC class I and II molecules and are sensitive to regulatory signals provided by cellular and humoral components of the Id-specific immune network (47). The majority of tumor cells in MM, however, are bone marrow-infiltrating myeloma plasma cells, which may not express surface Ig. Myeloma plasma cells secrete the idiotype M-com-

ponent and express cytoplasmic Ig, which carries Id determinants. It has been shown that cytoplasmic Ig in mouse B-lymphoma and plasmacytoma cells is processed intracellularly and that degraded Id peptides are presented on the cell surface in the context of MHC molecules (48). Moreover, myeloma plasma cells may express MHC class I antigens (49,50); adhesion molecules, such as CD44, CD56, CD54, and VLA-4 (51,52); signaling or costimulatory molecules CD40 and CD28 (50,53,54); and the Fas antigen (CD95) (55). Some of the plasma cells also express HLA-DR, CD80, and CD86 (50). Our study showed that myeloma plasma cells were able to activate alloreactive T cells and present the recalled antigens, purified protein derivative, and tetanus toxoid to autologous T cells (50). Therefore, it is conceivable that myeloma plasma cells may also be subject to immune regulation, at least by the cellular components of the immune network (47).

Id proteins are myeloma-specific antigens and should evoke an immune response in patients (56). To prove this point, various approaches have been used (57–60). The presence of Id-specific T cells in the peripheral blood of patients with MM or with the benign form of the disease, monoclonal gammopathy of undetermined significance (MGUS), has been studied by detecting Id-induced T-cell proliferation and cytokine secretion with the enzyme-linked immunospot (ELISPOT) assay. Our results showed that Id-specific T cells could be detected in 90% of patients with MM or MGUS (61–63) and these T cells responded to peptides corresponding to complementarity-determining region (CDR) I–III of heavy and light chain of the autologous Id protein (64,65). Furthermore, we demonstrated that Id-specific T helper 1 (Th1)-type cells (interferon [IFN]- γ - and/or interleukin [IL]-2-secreting cells) were significantly higher in patients with indolent disease (MGUS and MM stage I) than in those with advanced MM (stages II/III). In contrast, cells secreting the Th2-subtype cytokine profile (IL-4 only) were seen more frequently in patients with advanced MM (stages II/III) (66). Others have also reported a similar pattern of cytokine secretion (67). To examine whether Id-specific T cells can recognize myeloma cells, we generated Id-specific CTL lines from myeloma patients (68). The results showed that Id-specific CTLs not only recognized and lysed autologous Id-pulsed DCs but also significantly killed autologous primary myeloma cells (Fig. 1). The CTLs lysed the target cells mainly through the perforin-mediated pathway (data not shown). Consistent with these results, Li and coworkers (69) also showed that Id-specific CTLs, which consisted of both CD4+ and CD8+ T cells, lysed autologous myeloma cells, but not normal B cells or K562 cells. Thus, these studies provide a rationale for Id-based immunotherapy in MM.

Myeloma tumor cells may contain a multitude of tumor antigens that can stimulate an increased repertoire of antitumor T cells, possibly leading to stronger antimyeloma responses. To explore the possibility of using myeloma cells as the source of tumor antigens for immunotherapy in MM, we also generated autologous myeloma lysate-specific CTLs by culturing T cells with autologous DCs pulsed with myeloma tumor lysate (70). The CTL lines proliferated to autologous primary myeloma cells and DCs pulsed with autologous, but not allogeneic, tumor lysate (Fig. 2), and secreted predominantly IFN- α and tumor necrosis factor (TNF)- α , indicating that they belonged to the type-1 T cells (Th1 and T cytotoxic-1 [Tc1]). As depicted in Figure 3, CTLs had strong cytotoxic activity against autologous tumor lysate-pulsed DCs and primary myeloma cells. CTLs from some patients had no cytotoxic activity against Id-pulsed DCs but slightly lysed the allogeneic primary tumor cells, suggesting that Id was not necessarily

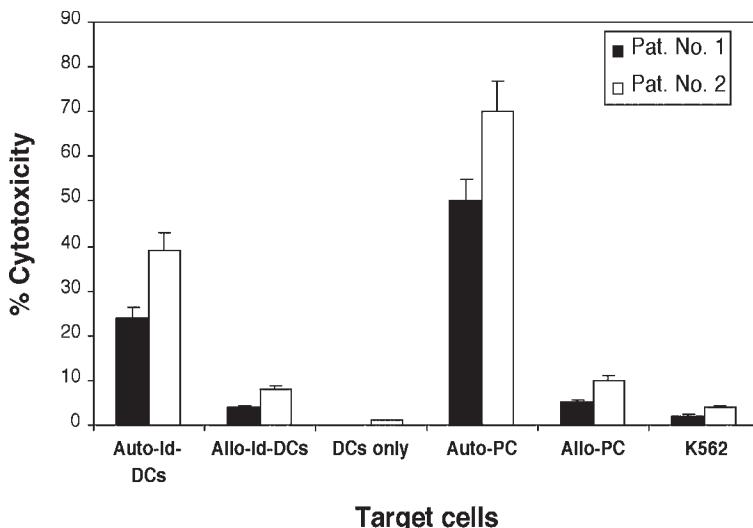


Fig. 1. Representative results depicting the cytotoxicity of Id-specific CTL lines generated from two patients against DCs pulsed with the autologous Id (Auto-Id-DCs), an allogeneic Id protein from the other patient (Allo-Id-DCs), unpulsed DCs (DCs only), or autologous primary myeloma cells (Auto-PC) or allogeneic myeloma cells (ALLO-PC), and K562. The effector-to-target cell ratio was 20:1.

a part of the lysate antigens and that there were shared tumor antigens among patients. No killing of autologous peripheral blood mononuclear cells (PBMCs), purified B cells, or Epstein-Barr virus-transformed B-cell lines was observed. Consistent with our results, a recent study also showed that myeloma bone marrow-derived T cells, after ex vivo stimulation with autologous tumor-loaded DCs, could kill autologous myeloma cells, but not nonmyeloma cells in the bone marrow, myeloma cell lines, DCs loaded with Id protein, or allogeneic myeloma cells (71). Collectively, these data demonstrate that CTLs induced by tumor lysate-pulsed DCs specifically kill autologous tumor cells, but not normal PBMCs or B cells, and provide a rationale for vaccination with tumor cell-pulsed DCs in myeloma patients.

3.2. Idiotype-Based Protein Vaccines for Myeloma

Our group at the Karolinska Institute, Stockholm, Sweden, was the first to introduce active immunization of myeloma patients with Id proteins (72,73). Having considered that immunotherapy may work better in immunocompetent patients with a low tumor burden, we targeted untreated patients with early disease. In our first pilot study, we recruited and immunized five previously untreated patients with stages I–III MM with the autologous Id protein precipitated in an aluminum phosphate suspension (72). In three patients, an anti-Id T-cell response, detected by enumeration of IFN- γ - and IL-2-secreting cells by ELISPOT assay, was amplified 1.9- to fivefold during the immunization. The number of B cells secreting anti-Id antibodies also increased in these three patients, and two of the three patients had a gradual decrease of blood CD19+ B cells. However, the induced T-cell response was transient and was eliminated during repeated immunization. The disease was stable in all patients, and no side effects or clinical response were noted.

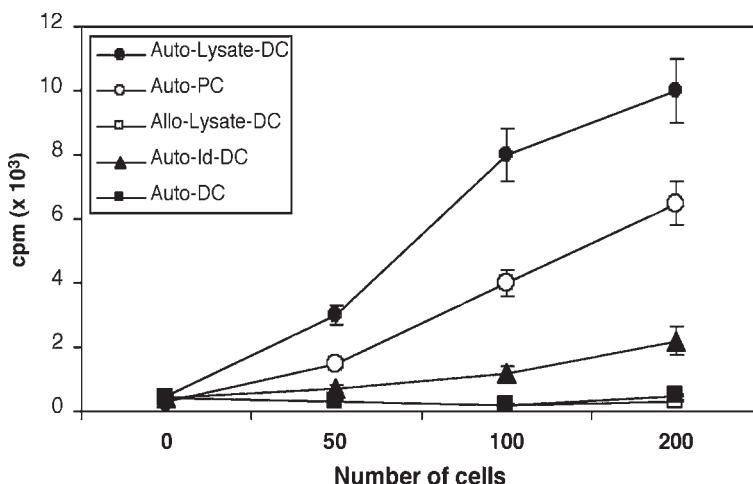


Fig. 2. Representative proliferative response (cpm) of a tumor lysate-specific CTL line from an MM patient in response to DCs pulsed with autologous tumor lysate (Auto-Lysate-DC) or Id protein (Auto-Id-DC), or autologous primary myeloma cells (Auto-PC). Controls include DCs alone (Auto-DC) and DCs pulsed in allogeneic tumor lysate from another patient (Allo-Lysate-DC).

In our second series of the study, immunization was performed by subcutaneous or intradermal injection of Id protein and GM-CSF (73). Five patients with IgG myeloma were treated, and an Id-specific type-1 T-cell response developed in all of them. The response involved both CD8+ and CD4+ subsets and was mainly MHC class I restricted. There was a transient rise in B cells producing IgM anti-idiotypeic antibodies in all patients. One patient had a clinical response, defined by a significant decrease in serum Id protein (from 20 g/L to 7 g/L) and normalization of serum Ig levels, which lasted for more than 1 yr after commencement of immunization. Although these studies involved a limited number of patients, the results clearly indicated that Id protein vaccination, especially in combination with GM-CSF, was able to induce specific anti-Id cellular and humoral immune responses, which were occasionally accompanied by a clinical response in treated patients.

Other clinical settings for immunotherapy could be minimal residual disease status achieved by high-dose chemotherapy and early host immunologic recovery following stem cell transplantation. These are supported by a study from Massaia and coworkers (74) showing that Id vaccination of myeloma patients with minimal residual disease was able to induce a strong Id-specific cellular immunity in many of the patients. In their study, 12 patients who had been treated with high-dose chemotherapy followed by stem-cell support received Id-KLH vaccines and a low dose of GM-CSF or IL-2. In most of the patients, the interval between the completion of prior high-dose therapy and vaccination was only 2–3 mo. Generation of Id-specific T-cell proliferative responses was documented in only two cases; however, a positive, Id-specific, delayed-type hypersensitivity (DTH) skin test reaction was observed in 8 of the 10 patients studied. The induction of humoral and cellular immune responses to KLH was observed in 100% and 80% of the patients, respectively, suggesting that the majority of patients shortly after high-dose therapy and stem-cell transplantation were already able to mount immune responses to KLH. Collectively, these results indicate that immunization of myeloma patients with the

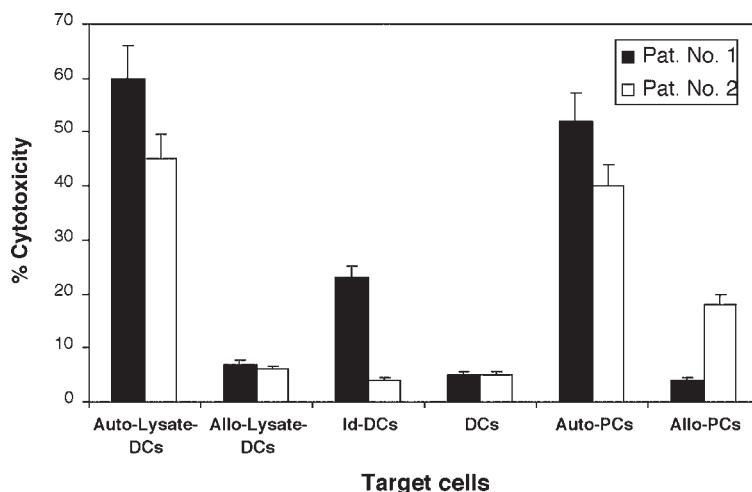


Fig. 3. Representative results showing the cytotoxicity of tumor lysate-specific CTL lines from two patients against DCs pulsed with autologous tumor lysate (Auto-Lysate-DCs), an allogeneic tumor lysate from another patient (Allo-Lysate-DCs), or autologous Id protein (Id-DCs), unpulsed DCs (DCs), and autologous (Auto-PCs) or allogeneic primary myeloma cells (Allo-PCs). The effector-to-target cell ratio was 20:1

autologous Id protein, together with GM-CSF, might be a promising immunotherapy method.

Id immunization may also be used in allogeneic bone marrow transplantation. Kwak and coworkers (75) immunized an HLA-identical sibling marrow donor with the patient's (recipient's) Id protein and showed that Id-specific T-cell immunity was successfully transferred to the recipient. The transferred anti-Id T-cell immunity was transient (<60 d), indicating that booster immunization of the recipient may be required to maintain the antitumor immunity.

3.3. Dendritic Cell-Based Vaccines for Myeloma

Preclinical studies have shown that DCs generated from myeloma patients were functional and could efficiently present Id determinants to autologous T cells (76,77). Compared with their progenitor monocytes, Id-pulsed DCs induced not only a stronger Id-specific T-cell response but also a predominant type-1 (IFN- γ) T-cell response (76). Both type-1 and type-2 (IFN- γ and IL-4) T-cell responses were noted when monocytes were used as the APCs. These results indicate that DCs pulsed with Id protein can be used to induce the type-1 anti-Id response in myeloma patients.

Wen and coworkers (78) reported vaccinating an MM patient with autologous Id protein-pulsed DCs generated from blood-adherent cells. Enhanced Id-specific cellular and humoral responses were observed in the patient. The immune responses were associated with a transient minor fall in the serum Id protein level. In their subsequent study, six additional patients were treated according to the same protocol (79). An immune response against Id was demonstrated in many of the patients. A minor clinical response (25% reduction in the M-component) was observed in one patient and stable disease in the remaining patients. Reichardt and coworkers (80) reported their experience with Id-pulsed DC vaccination in 12 myeloma patients after autologous peripheral blood

stem-cell transplantation. Their results were less compelling because only 2 of 12 patients mounted cellular Id-specific proliferative responses as the sole evidence for effective vaccination. Nevertheless, all myeloma patients could mount a strong anti-KLH response despite recent high-dose therapy. Similar results were also obtained in their subsequent study involving 26 patients treated on the same protocol (81). Although 24 of 26 patients generated a KLH-specific cellular proliferative immune response, an Id-specific proliferative immune response developed in only 4 patients. No clinical benefit was observed. These results suggest that DC-based Id vaccination is feasible after transplantation and can induce an Id-specific T-cell response in certain patients.

Other clinical trials of Id-pulsed DC vaccination in myeloma patients have been reported. Cull and coworkers (82) reported on their experience of vaccinating two patients with advanced refractory MM with Id-pulsed DCs combined with GM-CSF. An anti-Id T-cell proliferative response was detected in both patients, which was associated with IFN- γ production by the T cells. One patient also had an anti-Id humoral response. Titzer and coworkers (83) treated 11 patients with advanced MM with Id-pulsed, CD34+ stem cell-derived DCs and GM-CSF. After vaccination, 3 of 10 analyzed patients showed an increased anti-Id antibody titer, and 4 of the 10 patients had an Id-specific T-cell response measured by ELISPOT assay. Using partially purified DCs from unmobilized leukapheresis by gradient density centrifugation (Dendreon Corp., Seattle, WA) and ex vivo incubation with autologous serum containing Id protein as vaccine, Lacy and coworkers (84) treated MM patients with the vaccine 2.5–10 mo (mean, 3.4 mo) post-transplantation as a consolidation therapy. Among 28 patients who had residual disease at the initiation of DC vaccination and were evaluable for response, 8 responded (6 with complete or nearly complete remission and 2 with partial remission). However, whether the response was due to prolonged effects of high-dose chemotherapy and autologous transplantation or to DC vaccination was not determined. No immunological response was monitored or reported. Our groups at the Karolinska Institute (Yi et al., unpublished data) and the Myeloma Institute for Research and Therapy, Little Rock, Arkansas (Munshi et al., unpublished data) had also explored Id-pulsed DC vaccination in MM in a post-transplantation setting, similar to the studies mentioned above. Despite the use of maximal doses of Id-pulsed DCs (up to 5×10^7), only 10–30% of patients responded immunologically.

Collectively, the results of Id-pulsed DC vaccination have been disappointing: less than 50% of patients mounted an Id-specific immune response, and clinical responses have rarely been observed. In addition to the weak immunogenicity of Id protein, all of the studies mentioned above used Id-pulsed immature DCs as the vaccines administered intravenously to the patients (78–84). As we better understand the biology of DCs and immunotherapy, several possible explanations for this lack of response can be offered. First, the route of administration of DCs was not optimal. Several studies have shown that intravenous injection of DCs leads to accumulation of the cells in the lung, liver, and spleen during the first 24–48 h (85,86), whereas DCs injected subcutaneously migrate to the T-cell regions of draining lymph nodes and induce a strong protective immune response (85,86) or the type-1 T-cell response (87). Second, immature cells are potent at taking up antigen but less efficient at activating T cells; therefore, the cells may take up other antigens after administration and lose the expression of Id epitopes. In addition, monocyte-derived immature DCs are not stable and may differentiate back to macrophages when IL-4 and GM-CSF are withdrawn (88). Thus, by intravenous administration

Table 1
Induction of Anti-Id Immunological and Clinical Responses in Myeloma Patients After Id-Pulsed DC Vaccination

Responses	Pat 1	Pat 2	Pat 3	Pat 4	Pat 5
T-cell responses					
IFN- γ	+	+	+	+	-
IL-4	-	-	-	-	-
Proliferation	+	+	-	-	-
DTH reaction	-	-	-	-	-
B-cell response	+	+	+	+	+
Clinical response ^a	PR	SD	SD	SD	PD

^aPR, partial remission; SD, stable disease; PD, progressive disease.

of Id-pulsed immature DCs, the likelihood of the infused cells acting as potent DCs in presenting Id antigen and activating specific T cells is very low. Furthermore, two recent studies have shown that immature DCs, either used *in vitro* to stimulate T cells or injected *in vivo* in healthy donors, resulted in specific inhibition of antigen-specific response by inducing antigen-specific IL-10-producing T cells (89,90). Finally, patients were vaccinated shortly after completion of high-dose therapy or had a high tumor burden. Because these patients had a compromised immune system or bulky disease, DC vaccination would not likely induce an optimal immune activation that could lead to the control or eradication of tumor cells in such patients. Thus, DC vaccination protocols need to be optimized.

To improve the efficacy of DC vaccination in myeloma, we investigated the use of Id-pulsed mature DCs administered subcutaneously. In our study, five patients with stable partial remission following high-dose chemotherapy were vaccinated at least 4 mo post-transplantation (91). After four DC vaccinations, Id-specific T-cell responses, detected by ELISPOT (four patients) and proliferation (two patients) assays, were elicited in four patients and anti-Id B-cell responses in all five patients. The cytokine-secretion profile of activated T cells demonstrated a type-1 T-cell response. A 50% reduction in serum Id protein was observed in one immunologically responding patient and persisted for more than 1 yr; stable disease was noted in the other three patients. The remaining patient without an immune response to the vaccination experienced disease relapse (Table 1). These results are promising but preliminary because only five patients were involved in the study. Currently, we are performing a phase II study to evaluate the efficacy of intranodal DC vaccination in myeloma patients. Two groups of patients are targeted: group A includes patients with indolent or smoldering MM who will receive Id-pulsed, CD40 ligand (CD40L; Amgen Inc., Thousand Oaks, CA)-matured DC vaccines, and group B comprises patients with advanced MM who will receive tumor lysate-pulsed, CD40L-matured DC vaccines. To enhance efficacy, KLH is added to the vaccine, and a low dose of IL-2 is administered to patients after each vaccination. To induce optimal tumor reduction without compromising immunotherapy, advanced MM patients will be vaccinated up-front to generate Id-specific T cells, and blood T cells, which may contain specific T cells, will be collected. After high-dose chemotherapy and tandem autologous

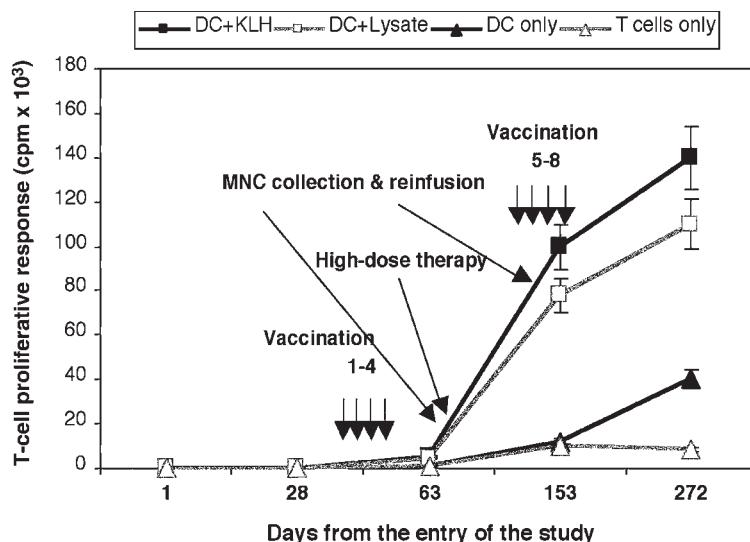


Fig. 4. Proliferative response of an MM patient receiving tumor lysate-pulsed DC vaccination and high-dose therapy (high-dose chemotherapy plus autologous transplantation) to KLH- and autologous tumor lysate-pulsed DCs increased substantially after vaccination. Mononuclear cells (MNC) were collected after the first series of DC vaccinations and reinfused into the patient after high-dose therapy.

transplantations, collected T cells will be reinfused, and four more DC vaccinations will be given to patients to boost specific immunity. So far, 12 patients have been enrolled (8 in group A and 4 in group B). As shown in our preliminary results (92), robust immune responses to vaccinations with autologous myeloma lysate-pulsed DCs can be induced in advanced-stage patients receiving DC vaccinations, high-dose chemotherapy, and tandem transplantations (Fig. 4).

DC vaccines can also be made in the form of fusion of tumor cells with DCs. The heterokaryons generated by tumor-DC fusion combines the machinery needed for immune stimulation with presentation of a large repertoire of antigens. Vaccination with fusions of tumor cells and DCs is an effective treatment in animal tumor models (93–95) and possibly in patients with metastatic renal carcinoma (96). In a murine plasmacytoma model, vaccination with DCs fused with mouse 4T00 plasmacytoma cells was associated with induction of antitumor humoral and CTL responses (97). Immunization with the fusion cells protected mice against tumor challenge and extended the survival of tumor-established mice without eradication of the tumor cells. Addition of IL-12 helped eradicate the established tumor. In a more recent study, human myeloma cells, either primary myeloma cells from patients or a myeloma cell line, U266, were fused to human DCs (98). It was demonstrated that fusions with mature rather than immature DCs induced higher levels of T-cell proliferation and activation, as assessed by intracellular IFN- γ expression, and stronger CTL activity against the tumor cells. On the basis of these results, a clinical trial was designed to evaluate the efficacy of vaccinating myeloma patients with a fusion of myeloma cells and autologous mature DCs (98).

3.4. DNA Vaccines for Myeloma

Various approaches to antitumor therapy are currently being explored that use both antigen-encoding DNA and noncoding nucleotides as components of gene vaccination (for a review, see ref. 99). These strategies include the construct that fuses an scFv incorporating both variable-region genes necessary to encode the Id determinants with fragment C of tetanus toxin (42), and gene transfer of cytokines or costimulatory molecules into myeloma cells by nonviral and viral vectors (100,101). In animal studies, DNA vaccination promoted specific immune responses and induced strong protection against B-cell lymphoma and myeloma (42–45). These strategies may have implications for immunotherapy in human diseases.

A phase I study had been completed to evaluate the feasibility and safety of vaccinating MM patients after high-dose chemotherapy with adenovector-engineered, IL-2-expressing autologous plasma cells (102). Eight patients were enrolled and vaccines were successfully made in six patients, who received from one to five subcutaneous injections of $3.5 - 9.0 \times 10^7$ cells/injection. Vaccines were well tolerated, with only minor systemic symptoms reported. Vaccination induced a local inflammatory response consisting predominantly of CD8+ T cells. However, no specific antitumor immune or clinical responses were noted. Hence, further studies of immunological and clinical efficacy are needed to examine the applicability of this approach to the treatment of patients.

4. VACCINES FOR LEUKEMIAS

4.1. Vaccines for Acute Lymphocytic Leukemia (ALL)

Clonal expansion of cells bearing rearranged antigen receptors is a hallmark of ALL of B- and T-cell lineages. In more than 30% of pre-B-cell leukemias, one of the two alleles is productively rearranged, and an in-frame Ig heavy chain polypeptide is expressed intracytoplasmically. In about one-half of these patients, the rearrangements are stable and therefore represent suitable targets for immunotherapy (103), as demonstrated by the successful induction of protective anti-Id B-cell response against the Id Ig expressed on the surface of B-cell lymphomas (11).

Several strategies for immunotherapy of ALL have been designed primarily to stimulate patients' immune system or to increase the sensitivity of leukemia cells to immune attacks (104). Examples of such approaches are the use of exogenous cytokines, mainly IL-2 and IFNs, but also IL-4, IL-7, IL-12, and granulocyte colony-stimulating factor (105). Another approach aims at mobilizing patients' antitumor immunity via recruitment and amplification of leukemia-specific responses with competent APCs. Here, either leukemia cells are modified to become immunogenic APCs, or patients' DCs are activated to trigger antileukemia T-cell responses (106). With the use of leukemia cells transfected with GM-CSF and CD80 genes as vaccines, a protective cell-mediated immunity could be induced in murine models of acute leukemias (103,107). These results suggest that gene transfer of GM-CSF and CD80 to leukemia cells can enhance the cells' immunogenicity and lead to the induction of a specific immunity against the tumor cells.

The use of CD40L has also been shown to be a valuable strategy to improve leukemia cell immunogenicity. In B-cell ALL, CD40L dramatically improved the immunogenicity of leukemia cells, which became competent APCs in both autologous and allogeneic settings (106,108). The ligation of CD40 on ALL cells resulted in upregulation of MHC

class I and II molecules and expression of CD80 and CD86 on the surface; it also enhanced the cells' capacity to stimulate the ex vivo generation of leukemia-specific CTLs from patients (106,108). These findings have clear clinical applications. Biagi and coworkers (109) transfected a human fibroblast cell line (MRC-5) with a clinical-grade adenovirus vector encoding the human CD40L gene. More than 90% of transfected cells expressed high levels of surface CD40L and could transfer CD40L to a patient's B-ALL tumor cells after coculturing. Now the B-ALL tumor cells expressed surface CD40L and, as a consequence, upregulated their surface expression of CD80, CD86, and ICAM-1; they also became potent APCs for stimulating an induction of leukemia-specific immune responses. Cotransfer of both CD40L and IL-12 genes to leukemia cells further enhanced cellular capacity to induce a stronger protective immunity against tumor cells in a murine study (110). On the basis of these findings, a phase I clinical study was designed and initiated to evaluate the feasibility, safety, and immunological efficacy of an IL-2- and CD40L-expressing tumor vaccine in patients with high-risk acute leukemia (111). The vaccine was made of autologous skin fibroblasts transduced with adenovirus vectors encoding human IL-2 and CD40L. In the preliminary report, nine patients (two adults and seven children with acute myeloid leukemia or B-cell ALL) in complete or partial cytological remission received up to six subcutaneous injections of their gene-modified CD40L and IL-2 fibroblasts, which were followed by injection of autologous leukemic blasts 2 wk later. No severe adverse reaction was noted, and most patients remained disease-free 1–31 mo after the first injection. An increase in IFN- γ - and IL-4-secreting cells reactive to their autologous tumor blasts was observed with the ELISPOT assay after three injections in these patients. Two of eight evaluable patients also had a humoral immune response to the tumor cells. Thus, the vaccine appeared to be safe and effective in inducing specific immune responses in treated patients.

A DC-based cell vaccine has also been piloted in acute leukemias. Fujii and coworkers (112) treated post-transplantation relapsed patients with acute leukemias with infusion of HLA-matched, allogeneic DCs that were pulsed with patients' irradiated tumor cells, together with ex vivo-primed autologous specific T cells. Four patients were enrolled, and DCs were generated from blood-adherent cells of HLA-matched donors. Autologous T cells were stimulated by HLA-matched allogeneic DCs that had been pulsed with patients' tumor cells. The specific T cells showed cytolytic activity against autologous tumor cells. Patients received, per intravenous injection, $2 - 20 \times 10^6$ irradiated, tumor-pulsed DCs weekly for up to 5 wk, together with primed T cells ($8 - 14 \times 10^6$ cells). Induction of a specific immune response was observed in three of the four patients, and a clinical response, shown as a decrease in circulating tumor cells, was observed in two of the patients, suggesting that treatment of relapsed leukemia patients with donor-derived, tumor-pulsed DCs and autologous, ex vivo-primed specific T cells might be effective as an adjunctive therapy.

4.2. Vaccines for Acute Myeloid Leukemia (AML)

AML cells can be made into cell vaccines for the disease. A strategy that has been extensively pursued is the differentiation or maturation of leukemia cells into DCs, which reflects the knowledge of DC development from primitive myeloid cells (32–34). A representative study by Choudhury and coworkers (113) demonstrated that the cytokine combination of GM-CSF and IL-4 together with CD40L or TNF- α induced primary AML cells from 18 of 19 patients to differentiate into DC-like cells that were able to

effectively stimulate the generation of leukemia-specific CTLs, when cocultured with autologous T cells. These CTLs lysed autologous leukemia cells but not normal cells. A more recent study from Harrison and coworkers (114) confirmed these results. In their study, blast samples from 24 of 40 patients with AML were differentiated into DC-like cells in the presence of GM-CSF, IL-4, and TNF- α . Most of the DC-like cells were able to activate allogeneic T cells and stimulate autologous T cells. Other reagents, such as stem-cell factor (115,116), transforming growth factor- β (116,117), FLT3 ligand (115), and cytokines IL-3 and IL-6 (118), have been used to optimize the conditions of generating AML DCs. As AML is a heterogeneous disease, further studies are required to predict the potential of leukemia cells from individual patients to differentiate into DC-like cells.

An alternative method is direct gene transfer of immunomodulators into AML cells as a means of improving their immunogenicity. These could be the costimulatory molecules and IL-12, which can facilitate T-cell priming; cytokines such as GM-CSF and IL-2, which trigger inflammation and recruit high numbers of professional APCs to the vaccination site; or GM-CSF, IL-4, and CD40L, which promote differentiation and maturation of leukemia cells into effective APCs (105). In murine models of AML, vaccination with irradiated leukemia cells transduced with CD80 (119); with GM-CSF, but not CD86-, IL-4-, or TNF- α -transduced AML cells (120); or with IL-12 elicited leukemia-specific protective and therapeutic immunity (121). Immunized mice showed no sign of systemic IL-12 toxicity, and their spleen histology was comparable with that of naïve mouse spleen (121). In a preclinical study, Koya and coworkers (122) transduced human ALL cells with third-generation self-inactivating lentivirus vectors expressing CD80 and GM-CSF. GM-CSF transduction and expression were associated with higher proliferation and cell viability, as well as enhanced capacity to induce allogeneic and autologous T-cell activation by transduced AML cells. Hence, these approaches might be feasible and applicable to immunotherapy in human disease.

In addition to blast-derived DCs, functional DCs can also be obtained from nonleukemic sources in patients with AML. Spisek and coworkers (123) recently reported on their experience with generating such cells from AML patients. In their study, mature, tumor-pulsed nonleukemic DCs were successfully generated from remission samples of all ($n = 10$) tested patients. These cells were used as APCs to induce leukemia-specific CTLs, which showed significant cytotoxic activity against autologous AML cells similar to that of CTLs stimulated by leukemic DCs. Thus, the induction of a leukemia-specific cytotoxic response by nonleukemic DCs cross-presenting apoptotic leukemic blasts offers a complementary approach to immunotherapy and has led to clinical trials. In their pilot study, Lee and coworkers (124) treated two patients with AML that had relapsed after autologous stem cell transplantation with DC vaccination. The vaccines were autologous CD14+ monocyte-derived DCs pulsed with AML tumor lysate. Patients received a mean of 7.8×10^6 DCs and 9×10^6 DCs, respectively. After vaccination, a positive DTH skin reaction and T-cell proliferation to tumor lysate-pulsed DCs were seen, although no improvement in patients' clinical status was observed. Further studies are needed to determine the appropriate dose of DCs and the most effective means of tumor antigen loading and presentation. Indeed, a recent study showed that DC-leukemia cell hybrids might be more potent at inducing specific CTL activity *in vitro* than DCs pulsed with either apoptotic leukemia cells or tumor-cell lysate (125).

4.3. Vaccines for Chronic Myeloid Leukemia (CML)

CML is characterized by a t(9;22) translocation that results in the expression of chimeric bcr/abl fusion oncoproteins necessary for oncogenesis. Like AML cells, CML-derived DCs show potential as tools for therapy. Leukemia cells of patients with CML will undergo substantial differentiation toward DCs and may be used to drive autologous T cells to acquire antileukemic cytotoxicity (for a review, *see* ref. 126). Early studies showed that both CD34+ bone marrow cells (127) and peripheral blood cells (128) could be differentiated into DCs after culturing with GM-CSF, TNF- α , and IL-4. These cells contained the CML-specific t(9;22) translocation as revealed by *in situ* hybridization, indicating that they were leukemic in origin. These leukemic DCs were able to stimulate the generation of specific CTLs that lysed autologous CML cells, but not normal blood or bone marrow cells (128). Addition of IFN- α to the culture may further improve the differentiation of CML cells into leukemic DCs (129). In a clinical study involving one patient with CML, infusion of leukemic DCs induced a vigorous CTL response *in vivo* that was accompanied by a decrease in the number of tumor cells in the peripheral blood and bone marrow (130). Nevertheless, two recent studies have shown that leukemic DCs from CML patients were functionally abnormal. Compared with DCs from normal donors, CML DCs had altered actin organization, reduced antigen processing, and impaired migration capacity (131). Furthermore, CML DCs displayed a reduced endocytotic capacity and deficiency in ex vivo maturation (132). These defects may be related to underlying cytoskeletal changes induced by the p210 (bcr-abl) fusion protein (131,132).

In CML, more than 90% of patients express the 210-kDa chimeric fusion proteins bcr2/abl2 or bcr3/abl2. Because bcr/abl chimeric protein is expressed only in CML cells, not in normal cells, the fusion sequence may act as a potential target for a T-cell-mediated immune response to CML. Within the fusion region of the bcr3/abl2 protein, different peptides have been identified that bind to HLA-A2, -A3, -A11, and -B8 (133,134). As CML DCs share a common progeny with leukemia cells, bcr/abl is constitutively expressed in these cells (135). The finding by Yasukawa and coworkers (136) that bcr/abl fusion protein-derived, peptide-specific CD4+ T-cell clones were able to augment colony formation by CML cells in a bcr/abl type-specific and HLA class II-restricted manner without addition of exogenous antigen suggests that CML cells can naturally process and present endogenous bcr/abl fusion protein to CD4+ T cells. Their subsequent study confirmed this finding (137). Thus, leukemia-derived DCs can be used as a vaccine to stimulate bcr/abl-specific CTLs. Alternatively, one can also prepare DC vaccines by transducing normal DCs with a virus vector expressing the fusion protein (138) or by pulsing DCs with bcr/abl peptides (139). Both strategies were efficient at stimulating specific CTL activities *in vitro*. Further studies are needed to evaluate their potential and efficacy as vaccines for treating CML patients.

As fusion proteins offer a good target for immunotherapy, vaccination with bcr/abl peptides has been explored in CML patients. Pinilla-Ibarz and coworkers (140) completed a phase I/II clinical trial to evaluate the safety and immunogenicity of peptide vaccination in 12 CML patients. Cohorts of three patients each received either 50 μ g, 150 μ g, 500 μ g, or 1500 μ g of total peptides (a mixture of equal amounts of four class I-restricted CML peptides and one class II-restricted peptide) mixed with 100 μ g of QS-21 as an immunological adjuvant. The vaccines were well tolerated. In three of the six patients treated at the two highest dose levels, peptide-specific T-cell proliferative

responses (three patients) and/or skin DTH reactions (two patients) were generated. Clinical response was not assessed because all patients remained on their current therapy while receiving the vaccine.

Leukemia cells also express other tumor-associated antigens that can be targeted for immunotherapy (for a review, *see ref. 141*). One antigen, proteinase 3, a serine proteinase present in the primary granules of neutrophils (142), can elicit a specific immune response in nonimmunized CML patients (143). PR1, a nine-amino-acid HLA-A2-restricted peptide derived from proteinase 3, is highly immunogenic (144). PR1-specific CTLs effectively lysed fresh, autologous CML blasts, and T-cell immunity to PR1 correlated with cytogenetic remission in CML patients treated with IFN- α or allogeneic marrow transplantation (145). On the basis of these observations, a phase I vaccine study was initiated at the M.D. Anderson Cancer Center to determine whether PR1 peptide could elicit CTL immunity in refractory leukemia patients (146). Nine patients were treated in cohorts of three at one of three dose levels of PR1 (0.25, 0.5, or 1.0 mg) in incomplete Freund's adjuvant and 70 μ g of GM-CSF every 3 wk in three subcutaneous injections. One patient with myelodysplastic syndrome (MDS), four with AML, and four with CML were enrolled, and two patients were in hematological or cytogenetic remission before the study. After vaccination, antineutrophil cytoplasmic antibodies did not develop in any of the patients, and there was no evidence of vasculitis. Two AML patients died of progressive disease. At each escalating dose level, none, one, and three of three patients, respectively, were in complete remission. PR1-specific CTLs were elicited in all four patients in complete remission. Three of these patients were induced into complete remission, including one patient with overt leukemia. Two patients with relapsed AML before vaccination attained cytogenetic remission after the second injection at dose levels 2 and 3. Thus, this study demonstrated that (PR1) peptide vaccination of leukemia patients could elicit highly active specific immunity against leukemia cells, inducing remission, and merits further studies to confirm the clinical efficacy of such an approach.

5. PERSPECTIVE AND CONCLUSION

Id protein has been used for the past 10 yr as the major antigen for immunotherapy in B-cell malignancies. As Id protein is a weak antigen, various strategies have been developed to enhance its immunogenicity, including the addition of or conjugation to KLH or GM-CSF and the use of other cytokines, such as IL-2, as immunomodulators and DCs as APCs (16,17,40,91). These approaches have shown immunological activity, but most of the treated patients did not benefit clinically. This may suggest that the elicited or enhanced immunity following Id vaccination is still too weak to cause significant tumor destruction, or alternatively immunization may generate a nonbeneficial immune response (e.g., a type 2 T-cell response; 147) that might enhance tumor B-cell growth and facilitate differentiation into plasma cell tumors (148,149). Ideally, a tumor-specific immunotherapy should induce or expand only the beneficial immune responses mediated by CTLs (Th1 and Tc1 subsets) that have sufficient cytotoxic effects toward tumor cells. Further studies are warranted to examine the interaction between B-cell tumors and Id-specific T-cell subsets so that a better understanding of the immune regulation mechanism in B-cell malignancies can be obtained. Furthermore, for B-cell malignancies and in particular, other hematologic malignancies, the discovery of other tumor antigens is

needed. For example, the Wilms tumor gene WT1 has been identified as a possible antigen in some leukemias.

The timing of immunotherapy is also crucial for its success. It is a consensus that immunotherapy may work better in immunocompetent patients with minimal tumor burden. In most, if not all, of the studies reported thus far, however, vaccination is administrated to patients shortly after high-dose chemotherapy when the immune system has not yet recovered. Although these patients are able to mount KLH-specific immune responses, it is highly possible that the responses are weak and not durable, compared with the same responses induced in immunocompetent individuals. This may partly explain the inability of the same patients to mount a tumor antigen-specific immune response to immunotherapy (tumor antigens are much less immunogenic than KLH), even with tumor antigen-pulsed DCs as the vaccine. Hence, it may be preferable to immunize patients up-front before chemotherapy to generate specific T cells *in vivo*, collect and freeze the primed T cells, and reinfuse them into the patients after the completion of high-dose therapy to partially restore the immune system and provide specific T cells. Additional vaccination can then be given to further expand tumor-specific immune responses in these patients.

DCs may be the best natural adjuvant for immunotherapy in human malignancies. Despite the success in animal and preclinical studies (30,31), however, the clinical evaluation of DC vaccination remains in its early phases, with a large number of technical variables awaiting *in vivo* testing before this approach is optimized. It is encouraging that early attempts at DC vaccination for tumor immunotherapy have demonstrated efficacy against several human tumors, including hematological malignancies. Further understanding of fundamental tumor immunology gained from well-designed clinical trials of DC-based vaccinations will improve the methods for inducing an effective antitumor immunity that will ultimately benefit clinically treated patients.

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Vaccines for the Immunotherapy of Prostate Cancer

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1. INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer and the second leading cause of cancer death in men in North America (1). Although locally confined disease is treatable, recurrent and metastasized prostate cancer is essentially incurable. Androgen ablation therapy often successfully, if temporarily, impacts the progression of advanced disease, as some prostate cancer cells are androgen-responsive (2–4). However, the majority of patients inevitably progress to incurable, androgen-independent disease (2,5–8). In addition to the acquisition of hormone refractivity, tumor heterogeneity in prostate cancer is also a major problem in the clinical management of this disease. Prostate tumors have many different populations of cancer cells expressing a variety of tumor-associated antigens (TAAs). In addition, the progression of prostate cancer from the hormone-naïve primary to an increasingly androgen-independent metastatic stage is associated with a number of molecular and genetic changes affecting the expression of specific TAAs on the cell surface. The challenge—not necessarily unique to prostate cancer—is that, in order to effectively treat the very heterogeneous prostate cancers, it is critical to identify novel markers and therapeutic targets in advanced prostate cancer and androgen-independent

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disease. Ideal targets for prostate cancer immunotherapy would include proteins that are: (a) highly expressed in metastatic prostate cancer cells; (b) not expressed, or expressed at very low level in normal tissues; (c) accessible to therapeutic modalities at the cancer cell surface; (d) not modulated by androgen. In the realm of prostate cancer, a number of promising immunotherapy targets have been identified, and are in various stages of preclinical development or clinical trials (9–17).

2. CELL-BASED VACCINES

In a recent clinical study, 60 patients with hormone-refractory prostate cancer were treated with a combination of irradiated allogeneic prostate cancer cells and the adjuvant SRL172 (18). Although there was no significant decline in prostate-specific antigen (PSA) from pretreatment levels, several patients had increases in cytokine and specific antibody production, as well as evidence of T-cell proliferation post-vaccination. Importantly, the vaccine was safe and well tolerated, suggesting that further exploration of whole-cell allogeneic vaccines for immunotherapy of less advanced prostate cancer may yield better clinical responses. A related, widely used approach to tumor vaccination involves irradiated tumor cells gene modified to secrete cytokines (19–22).

There are numerous cytokines in preclinical development, having demonstrated promise in *in vitro* experiments or initial animal models. Those tumor cell vaccines gene modified to produce granulocyte-macrophage colony-stimulating factor (GM-CSF) are one of the furthest along in clinical development of any prostate immunotherapy (23). GM-CSF is a monomeric glycoprotein that promotes the proliferation of macrophages, neutrophils, and eosinophils (24), and enhances dendritic cell (DC) migration, development, and longevity (25). Twenty-one patients with recurring micrometastatic prostate cancer were vaccinated with irradiated allogeneic cancer cells secreting GM-CSF (26). Subjects were given three to six vaccinations of 1×10^7 or 5×10^7 autologous prostate cells that were retrovirally transduced to produce GM-CSF (143–1403 ng/ 10^6 cells). The primary end points were toxicity, safety, and immune status. Side effects were moderate and transient, and included pruritis, swelling, and erythema at the injection site. Delayed-type hypersensitivity (DTH) reactivity against autologous tumor increased during therapy from two to seven of eight subjects, and displayed activation of both Th1 and Th2 T-cell responses. Prostate tumor cell-specific antibodies were elicited, demonstrating the induction of humoral immunity. One of the patients had a partial response lasting more than 7 mo. Fourteen of 21 patients had stable disease and 6 patients progressed. Importantly, 71% of the patients had a decreased PSA level compared with the one before vaccination. A phase II trial followed, for which 34 patients were evaluable (27). Participants received either low- (1×10^8 per vaccination) or high-dose (3×10^8 per vaccination) therapy with GVAX™ (Cell Genesys, South San Francisco, CA). Interim results indicated a dose-response relationship for time to clinical progression and survival. That is, 7 of 10 (70%) patients given the higher dose were still alive at the time of 2-yr follow-up, compared with 9 of 22 (41%) in the low-dose cohort. In addition, a longer median time-to-progression was observed in the higher-dose group (140 vs 85 d), as assessed by radiographic criteria. A phase III study with this vaccine is under way, as is a phase II study with new, irradiated, PC-3 and LNCaP cells modified to produce five- to 10-fold more cytokine. Such studies confirmed that GM-CSF-transduced allogeneic vaccines can break immune tolerance of prostate cancer and induce tumor-specific immunity, and has led to similar studies in melanoma and lung cancer patients with GVAX™ (28).

3. TELOMERASE

Telomerase, a cellular reverse transcriptase that maintains the ends of chromosomes (telomeres), is activated in the majority of cancers but not in normal adjacent tissues (29). More specifically, telomerase activity has been associated with prostate (and other) cancers, but not with normal prostatic tissue (30). These results suggest that telomerase activity may be a marker for prostate and other cancers.

Minev et al. identified two human leukocyte antigen (HLA)-A2-restricted peptides derived from human telomerase reverse transcriptase (hTRT), and induced hTRT-specific cytotoxic T lymphocytes (CTLs) *in vitro*. They demonstrated that the majority of normal individuals and patients with prostate cancer immunized *in vitro* against the hTRT-derived peptides could elicit tumor-specific CTLs. Most important, the CTLs of prostate cancer patients specifically lysed a variety of cancer cell lines, demonstrating immunological recognition of endogenously processed telomerase peptides (31). The ability to induce telomerase-specific T cells from the immune cells of patients with advanced cancer suggests they may be spared functional inactivation because of immunological ignorance (32).

It appears that telomerase-associated antigens also generate humoral immunity. In 2002, Lev and associates (33) described a group of human antibodies with antigen-restricted, major histocompatibility complex (MHC)-specific HLA-A2/hTRT epitopes on the surface of two established prostate cancer cell lines, PC-3 and LNCaP. These molecules exhibited the high-affinity binding characteristics of antibodies, yet retained one key characteristic of T-cell receptors: the capacity to distinguish native MHC-peptide complexes on cells.

4. PROSTATE-ASSOCIATED AND -SPECIFIC PROTEIN ANTIGENS

Prostate-associated and -specific protein antigens represent promising candidates for tumor-rejection antigens since they present organ specificity with little if any overlap of expression with nonprostatic tissues (34). Several antigens whose expression in tumors is largely limited to the prostate include PSAs (35–37), prostate-specific membrane antigen (PSMA) (38), prostate carcinoma tumor antigen (PCTA-1) (39), prostatic acid phosphatase (PAP) (40), putative tumor suppressor genes (41–43), prostate stem-cell antigen (PSCA) (44–46), and PARIS-1 (17). As is described below, the identification of tumor antigens and proteins involved in prostate cancer progression, and the promising vaccine studies in animal models, support the design and clinical testing of new prostate cancer vaccines.

Prostate cancer recurrence after standard therapy (surgery or radiation followed by hormone ablation) is an increasingly prevalent clinical problem. Such recurrence is often signaled by rising PSA levels after radical prostatectomy (35–36). PSA, commonly utilized as both a diagnostic indicator as well as a surrogate marker of response, is thought by many researchers to be a logical immunotherapeutic target, despite the controversy surrounding its clinical importance (47–48). Concerted efforts began in the mid-1990s at generating treatment benefit by directing immunity against PSA. The amino acid sequence of PSA was analyzed for theoretical binding motifs to common HLA haplotypes. Xue and coworkers initially demonstrated the ability of PSA-derived epitopes to generate antigen-specific CTLs from a healthy donor (49). Concern about tolerance to an antigen

shared between cancer and normal cells was alleviated upon demonstrating that PSA-based vaccination induced specific effector cells (50). The vaccine consisted of recombinant PSA with lipid A encapsulated in liposomes. This vaccination induced T-cell responses in 8 of 10 patients with prostate cancer, and was predominantly mediated by CD4+ T lymphocytes. Alexander and associates (51) could detect specific recognition of one PSA-derived peptide (PSA141–150). However, specific recognition of PSA peptides was not common as it was observed in only one of seven patients. The peptide-specific lymphocyte cell line did not recognize endogenous PSA, implying that the peptide may not be produced by prostate cancer cells producing PSA. Other groups have also produced PSA-specific T cells in vitro, although the difficulty in doing so might indicate few precursor cells exist (52–53). Such studies cloud the issue of whether such activity can be generated by active immunization strategies or can be therapeutic in men with established prostate cancer and clinically significant tumor burden.

Virus expression vectors can be used to drive the production of the antigen of interest, potentially enhancing its immunogenicity. In the field of prostate cancer, vaccinia-based vectors containing PSA are the furthest along in clinical development. Using a nonhuman primate model, Hodge and associates explored the immune responses to a recombinant vaccinia virus-expressed human PSA (rV-PSA) (54–55). Immunoglobulin M (IgM) antibodies were generated, as was long-lasting PSA-restricted T-cell reactivity. No adverse events were reported. In 1999, Sanda et al. (56) reported a clinical study undertaken to evaluate the safety and biologic effects of a vaccine using a vaccinia-based expression system to produce high levels of the target antigen (PROSTVAC). It was administered to six patients with post-prostatectomy recurrence of prostate cancer. Toxicity was minimal, and primary anti-PSA immunoglobulin G (IgG) antibody activity was induced after vaccinia-PSA immunization in one subject, although such antibodies were detectable in several subjects at baseline. Eder et al. (57) recently performed a similar phase I trial with a recombinant vaccinia virus expressing PSA in advanced prostate cancer patients. Thirty-three patients received the vaccine at 4-wk intervals for a total of three doses. Stable disease was reported in 14 of 33 patients for at least 6 mo. In five of the seven HLA-A2-positive patients the authors observed an increase of PSA peptide-specific T-cell frequencies. Interestingly, the T-cell increases were only observed after the first vaccination, and T cells did not further increase with subsequent vaccinations. Nonetheless, it further validated the safety and feasibility of the recombinant vaccine approach. Earlier stage studies are ongoing with fowlpox- and canary-pox-based expression vectors (58). These could prove effective, especially if used sequentially with vaccinia vectors in heterogeneous prime-boost combinations (58–59). Researchers have also attempted to increase antitumorigenicity by combining the aforementioned whole-cell vaccines with viral vectors (60–62).

These early clinical studies with synthetic and recombinant vaccines for prostate cancer are very encouraging. In contrast to other vaccine vectors, viruses elicit strong and long-lasting immune response, and are able to infect nearly all host cells, as well as to ensure intracellular translation, degradation, and efficient trafficking of peptide antigens to the cell surface. The potential drawbacks of the viral vectors are related to their safety and preexisting immunity, particularly to vaccinia virus and adenoviruses. However, the safety of the viral vaccines can be ensured by using nonreplicating, highly attenuated or genetically modified viruses, whereas the problem of preexisting immunity may be cir-

cumvented by the use of nonmammalian viruses, such as the avian poxviruses. Therefore, the use of recombinant viruses as cancer vaccines is very promising.

Murphy et al. assessed in a phase I clinical trial the safety of administering autologous DCs pulsed with HLA-A2-restricted PSMA peptides in 51 patients with advanced androgen-independent prostate cancer (63). Toxicity of the treatment was not observed, except for mild hypotension during the time of infusion. Patient clinical response was analyzed based on National Prostate Cancer Patient (NPCP) criteria and a minimum of 50% reduction of serum PSA levels. Seven partial responders were reported. Average PSA levels showed a significant increase in the nonresponder group, whereas a decrease was observed in the seven partial responders (64). The same group presented encouraging results in a phase II trial with prostate cancer patients. All study participants received six infusions of autologous DCs pulsed with the PSMA peptides at 6-wk intervals. With each infusion, half of study subjects received a 7-d course of subcutaneous injection of GM-CSF as systemic adjuvant. Based on the NPCP criteria and 50% reduction in PSA, 27% of the patients were identified as partial responders and 33% exhibited no significant change during the phase II trial (65). Twelve of 19 subjects (63%) with stage D₂ hormone-refractory metastatic prostate cancer survived for more than 600 d (median survival, 608 d) (64). No significant difference in clinical response was observed in patients who received subcutaneous GM-CSF injection with their DC/peptide infusions (66). To evaluate whether the responses were durable or not, study participants were afforded periodic follow-up evaluations after the conclusion of the study. A majority of the responders (11 of 19) (58%) were still responsive at the end of follow-up (67). This study suggests the majority of the responses identified in the various groups appear to be durable. The same researchers recently reported the immune monitoring of a phase II clinical trial in prostate cancer patients before and after immunotherapy with DCs exogenously pulsed with two PSMA-derived peptides (68). Clinical responses were strongly associated with two indicators of immunocompetence: skin test responses to recall antigens and cytokine secretion by T cells after nonspecific stimulation. These authors also reported that infusions with PSMA-pulsed DCs can be given with greater numbers of DCs and a lesser number of infusions, without immunogenicity being negatively impacted (69). However, it is critical to point out that the lack of a stringent record of any concomitant treatments sought by those on this vaccine makes its final evaluation difficult. A phase I/II trial with DCs exogenously loaded with recombinant, human PSMA protein was completed in 2002.

PAP is a prostate-specific isoenzyme among a heterologous collection of acid phosphatases secreted by prostatic cells. The immunogenicity of PAP-derived peptides has been only recently established (70). In a syngeneic murine model, it was shown that the preexisting tolerance to PAP could be surmounted and yet not provoke autoimmune prostatitis (71). It was used in two sequential phase I and phase II clinical trials in patients with hormone-refractory prostate cancer (72). Intravenous administration of peptide-pulsed DCs monthly for 3 mo resulted in T-cell proliferative responses to PAP in all patients. The vaccine, called Provenge™ (Dendreon Corp., Seattle, WA), consists of autologous DCs loaded *ex vivo* with a recombinant fusion protein consisting of PAP linked to GM-CSF. Twelve patients were treated in the phase I trial, 19 patients in the phase II trial. All patients tolerated the treatment well, with fever as the most common adverse effect (14% of the infusions). All patients developed T-cell proliferation

responses, and 38% of patients developed immune responses to PAP. Specificity of this therapy was demonstrated by the fact that treatment with Provenge™ did not increase the patient's response to the recall antigen influenza. Importantly, six patients had a decline in the PSA levels. There was a correlation between the development of an immune response to PAP and the time to disease progression.

PAP was also used to explore the potential role of xenoantigen immunization in prostate cancer patients. Fong et al. performed a phase I trial using DCs pulsed with recombinant mouse PAP as a vaccine (73). Twenty-one subjects were immunized twice with mPAP-loaded DC at a 4-wk interval. All patients developed T-cell immunity to mouse PAP, and 11 of the 21 patients also developed Th1 responses to the homologous self-antigen. Of note, 6 of the 21 patients experienced stabilization of disease as assessed by serum PSA and confirmed with radiographic imaging. This study represents the first demonstration that xenoantigen immunization can generate immune response against self-antigens in humans, resulting in a clinically significant antitumor effect.

Heiser et al. have shown that monocyte-derived DCs from prostate cancer patients transfected with PSA mRNA are capable of stimulating antigen-specific CTL responses *in vitro* (74). The same group subsequently performed a phase I trial to evaluate the safety and efficacy of this approach in patients with metastatic prostate cancer (74–75). This trial demonstrated that the administration of PSA RNA-transfected DCs stimulated the induction of PSA-specific T-cell responses without toxicity in all study patients. In addition, vaccination was associated with decrease in the log serum PSA slope in six of seven patients. A transient clearance of circulating tumor cells was also observed, suggesting some impact on tumor progression. This study provides evidence on safety and *in vivo* bioactivity of RNA-transfected DCs in patients with metastatic prostate cancer. Therefore, the use of DCs transfected with RNA-encoded antigens may allow stimulation of specific CTLs from peripheral blood mononuclear cells (PBMCs) of virtually all cancer patients independent of their MHC type, in contrast to the peptide-based vaccines, which are limited to certain patient subsets.

Mincheff et al. developed a protocol for *in vivo* transfection of DCs by naked DNA and adenoviral immunization and subsequently undertaken phase I and II clinical trials (76). Participants were immunized with the plasmid PSMA/CD86 encoding the extracellular portion of the PSMA and the costimulatory molecule CD86. Three months later, the patients were immunized with Ad5PSMA, a recombinant adenovirus encoding PSMA. All immunizations were well tolerated with no abnormal vital signs or laboratory findings. Although all patients showed a positive DTH reaction at the site of the plasmid application, no anti-PSMA antibodies were detected. Nine of the 12 patients with advanced local prostate cancer responded to combined hormone and immune treatment, whereas 6 of the 18 patients with distant bone metastases responded with reduction of bone pain and at least 50% fall in PSA (77). Although these trials showed promising results, future studies are needed to evaluate the benefits of this approach.

5. CARBOHYDRATE ANTIGENS

MUC1 is a highly glycosylated type I transmembrane glycoprotein with a unique extracellular domain consisting of a variable number of tandem repeats (VNTR) of 20 amino acids (PDTRPAPGSTAPPAHGVTS) (78). It was demonstrated that the MUC1 protein expression and secretion in cancer patients is associated with high metastatic

potential and poor prognosis (79–80). A MUC1-derived vaccine composed of a peptide conjugated with keyhole limpet hemocyanin (KLH) was injected with the immune adjuvant QS21 in patients with prostate cancer (81). All patients generated IgM and IgG response after three immunizations, and the rate of PSA increase diminished in some participants (82). These important clinical studies imply that MUC1 could be a suitable target for immunotherapy of prostate cancer.

In another clinical trial, patients were vaccinated with the hexasaccharide Globo H (83–84)—a hexasaccharide initially isolated from an established breast tumor cell line—conjugated to KLH (85). Although clinical response was not observed, a decrease in PSA slopes was noted for 6–9 mo post-vaccination. This vaccine was able to induce specific high titer of IgG and IgM antibodies against Globo H. Such antibodies were capable of generating antibody-dependent cell-mediated cytotoxicity (ADCC) (86). Globo H-derived vaccines have a potential for treatment of prostate cancer because they can enhance B-cell immunity.

A phase I trial exploring the immunogenicity of the ganglioside GM2 conjugated to KLH in patients with relapsed prostate cancer was reported in 2001 (86). Patients received the vaccine at weeks 1, 2, 3, 7, and 19. It did not produce any serious adverse events related to study drug, and all patients generated high-titer IgM and IgG antibodies with specificity for GM2. All patients in the study with metastatic disease—except for one with a single involved lymph node—showed radiographic and biochemical progression of disease. The lone patient with the single lymph node involvement had radiological evidence of decrease in lymph node size by 50%, as well as stabilization of PSA levels.

6. MONOCLONAL ANTIBODIES

Monoclonal antibodies (MAbs) mediate tumor killing by activating ADCC or the compliment fixation pathway. In the 1990s, the technology needed to engineer chimeric human/mouse and fully humanized mouse monoclonals was accomplished. Already, it has been clearly shown that such reagents largely or entirely avoid toxicities associated with human antimouse activity (HAMA). It was HAMA-related adverse events that prevented cancer treatment of the 1970s and 1980s from treatment benefit since effective dosages could not be achieved (87). Conjugation to highly potent radiolabels and toxins should increase tumor penetrance and resulting lethality (88). Moreover, MAbs can theoretically be used in concert with cellular vaccines, since antibody determinants and CTL epitopes are usually exclusive to the other (89).

In the area of prostate cancer, PSMA has been one of the most common targets of vaccines aimed at providing adjuvant humoral immunity. As a membrane-bound G-coupled glycoprotein (90–91), it is particularly amenable to such an approach. Several concurrent clinical trials are under way with any of several second-generation, humanized, and fully human monoclonal antibodies specific for protein conformational epitopes on various domains. Flow cytometric analysis of a range of fully human monoclonals indicated a strong specific binding to live prostate cells and, consequently, recognition of native epitopes (92). Another reagent, CYT-356, contains 7E11.C5, monoclonal reactive with an epitope on PSMA (93). Initially produced from a hybridoma from mice immunized with a human prostate adenocarcinoma cell line (90), CYT-356 is presently in clinical trial as a diagnostic imaging agent (94). When conjugated to an α -particle-emitting isotope, anti-PSMA antibody ($[^{213}\text{Bi}]J591$) produced a significant improvement

in tumor-free survival using an athymic nude mouse model (95). Since PSMA expression was detected in the neovasculature of many tumor types, its therapeutic mAb applications should greatly increase in the near future (96–97).

A progression of human trials has been conducted using CC49, a murine IgG1 antibody recognizing TAG-72. TAG-72, a tumor-associated mucin, is expressed in a variety of adenocarcinomas, including prostate, breast, colon, and pancreas (98). In 1994, a phase II study with ^{131}I -CC49 in 15 men with hormone-independent prostate cancer proved well tolerated, yet no objective responses were obtained (99). In order to promote cell-surface expression of tumor antigens, Slovin and associates (100) administered an interferon (IFN)- γ dose of 0.017 mg/m² for 7 d before ^{131}I -CC49 administration. Though no subject achieved a >50% PSA decrease, a few were classified as stable via radiographic criteria. By using IFN- α concurrently with ^{131}I -CC49, investigators avoided the marrow suppression commonly encountered when the radioconjugate was injected as a standalone agent. In both studies with adjunctive cytokine administration, thrombocytopenia was the serious adverse event leading to dose-limiting toxicity. Still, in those given IFN- α , some clinical improvement occurred: five of six participants reported a diminution of pain, and two of these had modest radiographic improvement (101).

An intriguing partnership of cellular- and humoral-based vaccines arose from the production of what are referred to as “bispecific” antibodies (bi-MAbs), usually a hybrid hybridoma cell line that secretes IgG class antibodies capable of focusing T-cell activity (102). For cancer treatment, such bi-MAbs are most often designed to recognize a tumor-related epitope and the CD3 or CD28 antigen on abnormal cells (103–104). Such reagents can redirect CTLs to cancer cells (105). The furthest along in clinical development for prostate cancer is MDX-H210, which recognizes the non-ligand-binding domain of FC γ R1a (CD64) and the extracellular domain of HER-2/neu. In a phase I study of pharmacokinetics, MDXH210 doses ranging from 1 to 8 mg/m² were well tolerated in patients with prostate, lung, and breast cancer. Biologic activity was shown by a decline in circulating HER-2/neu, as well as binding of the agent to circulating monocytes that induced cytokine secretion (106). The initial phase II trial combined MDX-H210 with GM-CSF (5 mg/kg/d), and showed more serious side effects resulting in three subjects withdrawing from the study: two with Grade 4 adverse events (heart failure and dyspnoea) and one with Grade 3 allergic reaction. Despite the observed toxicities, 35% (7 of 20) evaluable prostate cancer patients achieved a >50% decrease in PSA, whereas 58% (7 of 12) had improved pain scores (107). Therefore, additional studies are planned.

7. FUTURE DIRECTIONS

Prostate cancer remains one of the leading causes of death despite the fact that systemic chemotherapy and other treatment modalities usually induce a transient clinical response. Therefore, development of new treatment approaches, such as immunotherapy, is essential. The growing number of TAAs identified in prostate cancer becomes a solid basis for vaccine development. However, the antigenic profile of these tumors is very complex, and consists of many peptides originating from various classes of protein. This fact should be considered carefully in designing cancer vaccines. Most recent vaccine studies focused on class I-restricted antigens as targets for cancer-specific CTLs. The characterization of class II-restricted antigens as targets for CD4+ T-cell responses will allow

concurrent immunization with class I and class II epitopes in order to generate more potent immune responses. In any case, the ideal vaccine most likely will consist of a cocktail of tumor antigens or proteins. Another key factor is the dose of antigen and the speed of antigen release in the vaccine formulations. High doses of antigen released faster may induce T-cell tolerance (108). Immune tolerance may be owing to fast expansion and subsequent elimination of specific T-cell clones, or to apoptosis induced by repeated stimulation of already stimulated T cells in cell cycle (109). Therefore, it is essential to select as immunogens those epitopes against which tolerance has not been induced (110).

Future cancer vaccine strategies will most likely focus on more potent approaches for immunization. The use of the entire antigenic proteins might well be superior to peptide vaccines. A whole protein may provide several T-cell epitopes presented by different MHC molecules. An additional advantage of the whole-protein vaccines may be the induction of humoral immune responses (111). Immunotherapy of prostate cancer can be effective in eliminating micrometastases, decreasing the immunosuppressive effects of the chemotherapy or radiotherapy, and increasing the resistance to viral or bacterial infections frequently occurring in cancer patients. Treatment benefit may be achieved as a standalone agent, or in conjunction with surgery, radiotherapy, and/or chemotherapy.

Many challenges exist in the development of safe and effective vaccines for prostate cancer. Cancer cells can undergo genetic alterations that result in loss of antigen expression or loss of the ability to present the tumor antigens. Recent advances in the design of polyvalent vaccines targeting several antigens may solve this problem. In addition, the possibility to treat patients with vaccines earlier in the course of the disease and to combine vaccines with other treatment modalities may also improve the vaccine efficacy. Further understanding of the mechanisms of the antitumor immune responses will provide a basis for improvement of the cancer vaccine approaches in the future. As a result, immunotherapy may become a major treatment modality of prostate cancer.

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Vaccine Therapy for Breast and Ovarian Cancers

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1. INTRODUCTION

Breast and ovarian cancers are ideal cancer vaccine targets for several reasons. Though perhaps not as immunogenic or responsive to immunotherapies as melanoma or renal cell carcinoma, evidence of endogenous immune responses exists in many patients with breast or ovarian cancer. A number of shared antigens or potential vaccine targets also exist between these two malignancies. Both tissue and tumor-associated antigens are known and, as both organs are dispensable, breaking self-tolerance to tissue-specific antigens is feasible. Additionally, adjuvant treatment for early-stage disease in breast and ovarian cancer is common. Since it is unlikely that cancer vaccines will eradicate large tumor burdens, vaccination of patients with low tumor burden will probably be most beneficial. Patients with breast cancer are often treated in the adjuvant setting when they have no measurable disease. Ovarian cancer patients are also typically treated aggressively after presentation and can often be rendered disease-free or with minimal measurable disease. Both groups of patients also have a significant and fairly predictable rate of relapse. Patients with breast and ovarian cancer also tend to be younger, healthier, and less immunocompromised by their cancer therapies than many other cancer patients. These diseases also affect large numbers of patients. Breast cancer remains the second

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leading cause of cancer deaths for American women, whereas ovarian epithelial cancer ranks fifth (1). For these reasons, breast and ovarian cancers are excellent targets for cancer vaccine development with readily available patient populations for testing new vaccine approaches.

Not surprisingly, breast and ovarian cancers have been targeted in several cancer vaccine trials to date. These trials have generally been small, published or presented only in abstract form, and disappointing with few clinical responses and only hints of immunologic reactivity reported. Thus, despite validated tumor-associated antigens (TAAs) for targeting, optimal patients to treat, and multiple different vaccine approaches attempted, vaccine trials in breast and ovarian cancer have still not produced consistently effective and/or specific antitumor immune responses.

2. ENDOGENOUS IMMUNE RESPONSES IN BREAST AND OVARIAN CANCERS

Initially pathologists were the first to suggest that intrinsic antitumor immune response could be generated *in vivo* in breast cancer. Black described lymphoid infiltration of primary breast tumors and sinus histiocytosis of regional lymph nodes (2). Tumor-infiltrating lymphocytes (TILs) have been identified in ovarian adenocarcinoma tissue as well as peritoneal malignant ascites (3–5). The isolation of TILs with activity against specific TAAs has allowed characterization of specific antigens.

For example, MUC-1 is a transmembrane glycoprotein expressed on the apical surface of normal glandular epithelial cells. It has multiple tandem peptide repeats that offer potential antigen sources for dendritic cell (DC) presentation. MUC-1 is overexpressed in adenocarcinomas including breast, pancreatic, and ovarian. When expressed on cancers, MUC-1 tends to be aberrantly glycosylated and expressed on the entire cell surface, rather than luminally as on normal cells (6,7). MUC-1 overexpression is also correlated with progression of ovarian cancer (8). MUC-1-specific cytotoxic T lymphocytes (CTLs) have been isolated from involved lymph nodes (9), ascites (10–12), and tumor tissue (13) in ovarian malignancies, as well as in draining lymph nodes in pancreatic and breast cancer patients (3,14). Because of its high surface-expression levels, aberrant tumor-associated glycosylation resulting in exposure of normally masked peptide epitopes, and broad expression across several tumor types, MUC-1 is a favored target in cancer vaccine trials.

Though most clinical trials attempt to generate cellular immunity, there is perhaps stronger evidence of endogenous humoral antitumor immune responses. Circulating levels of immunoglobulin G (IgG) and immunoglobulin M (IgM) antibodies complexed to MUC-1 have been demonstrated in the sera of breast and ovarian cancer patients (15,16). Although MUC-1 antibodies can also be demonstrated in pregnant women (17), over 50% of ovarian cancer patients have measurable levels of circulating MUC-1 antibodies that are typically at higher levels than those observed in control and pregnant subjects (18).

In breast cancer, immune activity has correlated with clinical outcomes in several studies. Histologic lymphoid infiltrates in primary breast tumors have been associated with improved survival (19). Elevated anti-MUC-1 IgG and IgM levels in stage I and II breast cancer patients have also predicted improved disease specific outcomes (20). Antitumor antibodies detected pretreatment in early-stage breast cancer patients have also

Table 1
Nonspecific Immune Modulators

Citation	Phase	N	Disease	Stage	Vaccine Approach	End Points	Toxicities
(25)	III	238	Breast	II–III NED	FAC ± BCG	No difference in survival, DFS, RR	BCG dermal toxicity
(24)	III	395	Breast	IV	CMF, CAF, or CAFVP ± BCG	BCG offers no survival benefit	BCG dermal toxicity
(27)	III	133	Breast	IV	FAC ± pseudomonas vaccine	No difference in survival, DFS, RR	1/65 anaphylaxis 33/65 skin reactions 25/65 dose reduced 8/65 stopped early

NED: no evidence of disease; FAC: 5-fluorouracil, adriamycin, cyclophosphamide; BCG: bacille Calmette-Guérin; CMF: cyclophosphamide, methotrexate, 5-fluorouracil; CAF: cyclophosphamide, adriamycin, 5-fluorouracil; CAFVP: cyclophosphamide, adriamycin, 5-fluorouracil, vincristine, prednisone; DFS: disease-free survival; RR: relapse rate

been shown to protect against distant metastatic spread (21). In stage IV patients, the presence of circulating antibodies directed against undetermined antigens with reactivity against random peptide phage display libraries, also has been correlated with improved survival (22), even though the antigen is yet unknown. Thus, there is strong evidence that specific antitumor humoral and cellular responses are generated in breast and ovarian cancer patients. Whether these immune responses actually play an important role in controlling disease or are surrogate markers of host immune competency that favor survival remains a research question.

3. EARLY VACCINE APPROACHES

3.1. Nonspecific Immune Stimulation

In the earliest immunotherapy approaches, immune adjuvants were combined with standard chemotherapy in an attempt to nonspecifically stimulate antitumor immune responses (*see Table 1*). After initial phase I trials reported that bacille Calmette-Guérin (BCG) added activity to adjuvant chemotherapy for breast cancer (23), two large multicenter phase III trials were performed to confirm these results (24–26). Budzar et al. compared 238 breast cancer patients treated adjuvantly with 5-fluorouracil/adriamycin/cyclophosphamide (FAC) with or without BCG (25). No differences in survival, disease progression, or relapse rate were noted between the BCG-treated and -untreated groups. Aisner et al. confirmed these negative findings in a larger trial of 395 patients with metastatic breast cancer (24). In both studies, significant dermal toxicity from the BCG injections was observed.

Another nonspecific immune modulator, killed heptavalent pseudomonas, was tested in a randomized phase III trial of 133 patients with metastatic breast cancer receiving FAC chemotherapy (27). The pseudomonas vaccine was also associated with significant toxicity, including local skin reactions and anaphylaxis, without evidence of improved

Table 2
Adoptive Therapy

Citation	Phase	N	Disease	Stage	Vaccine Approach	End Points	Toxicities
(28)	I	17	Ovary	IV	Adoptive transfer of expanded ip TIL followed by cyclophosphamide or cisplatin	1/17 CR 6/77 PR	Chemotherapy related
(29)	I	11	Ovary	IV	Adoptive transfer of expanded ip TIL + rIL-2 vs rIL-2 alone	36% with decreased CA-125, tumor regression, or stabilization	Peritonitis
(30)	I	20	Ovary	IV	IP rIL2 or rIFN γ with or without expanded TIL	3/20 PR increased MHC I and II	Abdominal pain

ip: intraperitoneal; TIL: tumor-infiltrating lymphocyte; CR: complete response; PR: partial response; rIL-2: recombinant interleukin-2; rIFN γ : recombinant interferon- γ ; MHC: major histocompatibility complex.

clinical activity observed. Thus, after initial encouraging results in phase I trials, confirmatory clinical activity has not been observed in larger randomized phase III trials and the use of nonspecific immune adjuvants alone or combined with standard chemotherapy regimens has been largely abandoned.

3.2. Adoptive Immunity

Improvements in T-cell culturing methods permit ex vivo expansion of a patient's TILs and the generation of high numbers of presumably tumor-reactive T cells. These cells can then be reinfused into patients where they would hopefully house to tumor sites and incite immune-mediated tumor death. This approach, known as adoptive immune therapy, has been tested in several clinical trials. Ovarian cancer is a natural target for adoptive immunity trials, as the high prevalence of ascites in these patients allow for easy and successful TIL isolation and ex vivo expansion. In addition, preclinical data have confirmed in vitro antitumor effects of TILs isolated from malignant ascites of ovarian cancer patients. Tumor-associated lymphocytes (CD3+/CD4+ and CD3+/CD8+ cells) have been successfully propagated in vitro in the presence of moderate concentrations of interleukin (IL)-2, with the propagated cells demonstrating preferential major histocompatibility complex (MHC)-restricted cytolytic activity against autologous tumor lines (11). The addition of IL-4 and tumor necrosis factor (TNF)- α to IL-2, further improved TIL cytotoxic activity (12). Based on the amplified killing observed with the use of cytokine cocktails, IL-2, IL-4, and TNF- α , and the clinical activity of TILs plus IL-2 therapy in melanoma trials, several clinical trials were initiated (*see Table 2*) (28).

In a phase I study performed at M. D. Anderson Cancer Center, eight patients with advanced ovarian cancer were treated with intraperitoneal (ip) TILs expanded ex vivo ($10^{10} - 10^{11}$ cells) with IL-2, followed by bolus ip IL-2 (29). Three patients received intraperitoneal IL-2 alone. Four patients treated with TILs plus IL-2 gained clinical

Table 3
Intact Irradiated Cell Vaccines

Citation	Phase	N	Disease	Stage	Vaccine Approach	End Points	Toxicities
(26)	III	131	Breast	II	CMF vs CMF + BCG vs CMF + irradiated allogenic tumor vaccine (three cell lines)	No difference in survival, Closed early due to hepatitis B infections in 14/41 pts receiving vaccine	Hepatitis B infection BGC dermal toxicity
(31)	I	40	Ovary	IV	Intraperitoneal and intrapleural irradiated oncolysate with influenza virus	23% clinical responses 3–19 mo duration of response	Fever, anorexia, abdominal pain
(32)	I	42	Breast	III–IV	Weekly subcutaneous multiantigen vaccine with autologous cancer cells, allogenic cells (MCF-7), and CA-15.3 CEA, and CA-125 proteins	2/42 with PR 7/42 with stable disease increased T-cell proliferation	None reported

CMF: cyclophosphamide/methotrexate/5-fluorouracil; BCG: bacille Calmette-Guérin.

benefit as determined by ascites regression, CA-125 stabilization, and/or tumor stabilization. There were also substantial increases in peritoneal cytokine levels (TNF- α , interferon [IFN]- γ) in these four patients. The most significant toxicity was peritonitis. Aoki et al. also reported high response rates (80%) to adoptive transfer of autologous TILs in combination with chemotherapy (cisplatin or cyclophosphamide). However, the small sample size, lack of control group, and concurrent use of effective systemic chemotherapy complicates interpretation of the data and the role of the TILs in the reported responses. Freedman et al. reported increased intraperitoneal human leukocyte antigen (HLA) class I and II expression after ip administration of recombinant IL-2 and IFN- γ (30). Tumor regression was observed in 3 of 20 patients after administration of the cytokines alone or coadministered with TILs. The tremendous labor, costs, and difficulty associated with isolating and expanding TILs ex vivo, along with the low response rates observed, has led to the abandonment of this approach except in a few research centers.

3.3. Intact Irradiated Tumor Cell Vaccines

Because few TAAs were known, early studies of cancer vaccines utilized irradiated tumor cells as the immunogen (*see* Table 3). Whole-tumor-cell vaccines also circumvent the difficulties of predicting immunogenic peptides or proteins and ensure a multitude of

TAAs will be included in the vaccine preparation. Irradiating the cells prevents tumor cell division and also may increase the expression of intracellular immunogenic antigens and heat shock proteins that may further augment effective antigen presentation and stimulation of the host immune system. In the 1980s, Freedman et al. administered allergenic oncolysates from two ovarian cancer cell lines infected with influenza virus intraperitoneally or intrapleurally to 40 patients with advanced ovarian cancer (31). Twenty-two percent of the patients had evidence of clinical response; 20% had ascites regression; and 6% had decreased measurable partial responses. The duration of responses ranged from 3 to 19 mo.

In a phase III trial, stage II breast cancer patients were randomized to either cyclophosphamide/methotrexate/5-flourouracil (CMF) chemotherapy alone, CMF plus BCG, or CMF plus BCG with irradiated allogeneic tumor cells from three breast cancer cell lines (26). No differences in breast cancer survival or response rates were noted with the addition of the allogeneic tumor vaccine. However, 14 of 41 (34%) patients who received the tumor cell vaccine developed hepatitis B infection. The high rate of hepatitis B infection closed the trial early and warned of the potential serious infectious risks associated with allogeneic approaches.

A recent study by Jiang et al. evaluated a combination cellular and antigen vaccine, which utilized irradiated allogeneic breast cancer cells (MCF-7), autologous breast cancer cells, several TAAs including CA 15-3 (MUC-1), CEA, and CA-125, along with small doses of the immunostimulatory cytokines IL-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (32). In this “kitchen sink” approach to vaccination, patients received six subcutaneous injections of the vaccine at weeks 1,2,3,7,11, and 15. Serious adverse events were not observed in 42 treated stage I-IV breast cancer patients though fatigue and injection site reactions were common. Two clinical responses were reported, but were not associated with decreases in their serum tumor markers, including CA 15-3 or CA-125, and tumor measurements were not provided. The only method used to quantitate immune responses was an increase in lymphocyte proliferation pre- and post-vaccination to the allogeneic, autologous tumor cells, or individual proteins. Though an increase in lymphocyte proliferation was seen in most patients to autologous tumor cells and CEA, CA-125, and CA15-3 proteins, a statistically significant increase in proliferation was surprisingly not observed to the allogeneic cell line and changes in proliferation were not related to clinical responses. Changes in the serum tumor markers, CEA, CA15-3, and CA-125, were also not observed in response to the vaccine. Thus, little beyond the safety of the vaccine can be stated with certainty.

4. TUMOR-ASSOCIATED ANTIGEN VACCINES

Many shared TAAs have been identified in ovarian and breast cancer patients (*see* Table 4). The list of potential antigens includes: cell-surface proteins that are overexpressed and released in the circulation by the cancer cells (MUC-1, HER-2/neu, CEA, CA-125, Ep-Cam), overexpressed genes (*p53*), specific or aberrantly glycosylated carcinoma-associated proteins (Sialyl-Tn, Tn, Globo H, Lewis^y [L^y]), and proteins whose expression is largely restricted to tumors (cancer testis antigens- NY-ESO-1, MAGE proteins). In breast cancer, the expression of HER-2/neu, MAGE-A, and Ep-CAM predicts clinical outcome and tumor behavior (33–35). Targeting proteins that are necessary for metastasis or tumor growth are attractive for vaccine development as this ensures the

Table 4
Breast and Ovarian Cancer Tumor-Associated Antigens

Cell-surface proteins	MUC-1 HER-2/neu CEA CA-125 EpCam
Mutated genes	P53
Glycosylated protein epitopes	Siayl-Tn (STn) Tn Thomasen-Freidrich (TF) Globo H Lewis ^y
Cancer-testis antigens	NY-ESO-1 MAGE, BAGE, LAGE SCP-1 SSX-2

tumor cell will not escape immune clearance by expression of the targeted antigen. The most widely studied immunogens in breast and ovarian cancer vaccine trials are derived from the MUC-1 and the HER2/neu proteins. CA-125 has been used as an immunogen in many ovarian cancer vaccines. Current trials are utilizing wild-type p53 peptides as an immunogens. Although CEA, CA-125, PSA, Ep-CAM, and MAGE-A proteins have been identified on breast cancer cells and are therefore potential TAAs, their use in breast-vaccine trials has been minimal to date.

4.1. MUC-1

MUC-1 is the most common human mucin and is found on a myriad of glandular epithelium (breast, ovary, colon, pancreas, prostate, gastric, lung). It is normally expressed on the luminal side of the epithelial lining and its function is still unknown. It is a large protein (40 kD), which contains multiple tandem peptide repeats of a 20-amino acid sequence that transverses the cell membrane multiple times (6,7). It also has numerous carbohydrate side chains. Both the tandem peptide repeats and carbohydrate side chains can act as immunogens in vitro. MUC-1 is usually masked from the immune system though anti-MUC-1 antibodies can be isolated from pregnant and even normal patient serum, as transitory disruption of glandular epithelium allows MUC-1 to enter the circulation, and thus become immunogenic. As parity increases, the number of MUC-1-specific circulating T cells also increases, as measured by in vitro T-cell stimulation by synthetic MUC-1 peptides (17,36). Significant disruption of the epithelium can also occur in other disease states. For example, patients with active ulcerative colitis may also demonstrate high levels of circulating anti-MUC-1 IgG through this mechanism (37). Burcell et al. developed monoclonal antibodies, HMFG-1 and HMFG-2, which recognize exposed epitopes on MUC-1, which are present in higher concentrations in breast cancers compared with normal breast tissue (6). There is considerable evidence for MUC-1-induced immune responses in patients with breast and ovarian cancers, as outlined earlier. Though MUC-1 is expressed on normal tissues, self-recognition does not

appear to be detrimental. Breast and ovarian cancer patients with demonstrable endogenous MUC-1 immunity have improved disease outcomes without increased autoimmune symptomatology (20,21).

To date, the majority of MUC-1 vaccine trials have been small phase I clinical trials evaluating safety and monitoring antitumor immunity at the cellular (CTL) and humoral levels as the primary end point. Few data on clinical outcomes are available as patients without measurable disease (NED or no evidence of disease) have predominantly been treated (*see* Table 5).

The most successful MUC-1 vaccine trials have used immune adjuvants, including keyhole limpet hemocyanin (KLH) and QS-21 to generate measurable immune responses (38,39). Development of anti-MUC-1 IgG was demonstrated in 15 immunized breast cancer patients who received intradermal vaccination with a 30-amino acid MUC-1 peptide–KLH conjugate plus QS-21. Toxicities were minor and included local skin reactions, flu-like symptoms, and mild leukopenia. The peptide–KLH conjugate plus QS-21 induced both IgM and IgG subclass antibody responses, but no evidence of cytotoxic or T-cell immunity was found by either limiting dilution chromium release assays or delayed-type hypersensitivity (DTH) skin reactions to MUC-1 (40,41). Interestingly, several of the patients with high post-vaccination IgG titers did not react against MUC-1-expressing breast cancer cell line, MCF-7, *in vitro* suggesting that the immunodominant epitope in this vaccine may not be exposed to the humoral immune system *in vivo*. In these trials, all treated patients were NED, often on concurrent hormonal therapies, and without significant elevations of known tumor markers. Thus, the role of elevated IgG or IgM anti-MUC-1 antibodies in protecting against breast cancer progression cannot be determined from these trials.

In another small trial of 16 patients with metastatic breast cancer who were immunized with a 16-amino acid MUC-1 peptide conjugated to KLH plus DETOX™ adjuvant, high-titer antibodies were predominantly induced against KLH rather than MUC-1 (42). In direct contrast to the work described above, a cellular immune response was measurable in 9 of 11 tested patients with 7 of these responses MHC class I restricted. However, baseline CTL activity was not obtained in these patients and clinical relevance is unknown, as tumor response data were not presented in the manuscript.

There are several studies that support induction of T-cell-mediated immunity with MUC-1 peptide vaccines. Gyodos et al. reported tissue biopsy evidence of an intense T-cell infiltration (DTH response) in 37 of 63 treated patients with breast, colon, or pancreatic carcinoma following intradermal vaccination with a 105-amino acid synthetic mucin MUC-1 peptide plus BCG (43). In this study, a two- to fourfold increase in mucin-specific CTLs were also observed, though the methodology for this testing was not defined.

In an Australian study, 25 patients with stage IV breast and gastrointestinal cancer were vaccinated with a 120-amino acid MUC-1 peptide chemically conjugated to mannan, a mannose containing polysaccharide (44). Animal studies had suggested that coadministration of peptides with mannan markedly increased immunogenicity. Patients were treated with increasing doses (from 10 to 500 µg) of mannan-MUC-1 intradermally. Anti-MUC-1 IgG was found in 13 of 25 patients tested. Higher titers of antibody were generated in patients vaccinated with >400 µg of antigen suggesting a dose response. T-cell proliferation to *in vitro* stimulation against MUC-1 peptides was observed in 4 of

Table 5
MUC-1 Vaccine Trials

Citation	Phase	N	Disease	Stage	Vaccine Approach	End Points	Toxicities
(49)	II	12	Breast	IV NED	STn-KLH + DETOX™ id + IV cyclophosphamide	12/12 w/ anti STn IgG & IgM Ab complement-mediated cytotoxicity 2/12 with PR 1/12 with stable disease at 10 mo	Mild skin reactions
(43)	I	63	Breast, GI pancreatic	Not reported	MUC-1 peptide + BCG id	55/63 w/T-cell infiltration in Bx	Skin breakdown
(51)	II	85	Breast Ovary Colon	IV	STn-KLH + DETOX™ id	7/22 w/increase in mucin-specific CTL 83/85 w/anti-STn IgG & IgM higher anti-STn titers correlated w/ survival in breast & colorectal cancer	Minimal
(50)	II		Colon Breast	IV	STn-KLH + DETOX™ id, ± iv or oral CTX	higher anti-STn titers w/IV CTX trend toward better survival ($p =$ 0.00176) with iv CTX vs other arms	Minimal
(44)	I	25	Breast, GI	IV	MUC-1 peptide + mannan id	4/15 w/T-cell proliferation 2/10 w/CTL response 13/25 w/increased anti-MUC1 IgG	None reported
(42)	I	16	Breast	IV	KLH-MUC-1 peptide + QS-21 id	16/16 w/anti-KLH titer 3/16 w/anti-MUC-1 titer 7/11 w/CTL response	None reported
(40)	I	6	Breast	IV NED	KLH-MUC-1 peptide + QS-21 ID	16/16 w/IgG and IgM response	None reported
(52)	I	40	Breast Ovary	III/IV	STn-KLH + DETOX™ id, after stem-cell transplant	Trend toward increased survival in patients w/+ immune response	Mild skin reactions
(46)	I	10	Breast	I-IV	KLH-MUC-1 peptide + QS-21 id	Increased anti-MUC1 Ab; CTL against MUC-1 cell lines	None reported
(39)	I	24	Breast	II-IV	Glycosylated KLH-MUC-1 peptide + QS-21 id	Ongoing analysis	Skin breakdown
(41)	I	9	Breast	III-IV NED	KLH-MUC-1 peptide + QS-21 id; + Tamoxifen	9/9 w/increased IgG & IgM Ab; no CTL responses	Flu-like sx Skin breakdown
(47)	I	6	Breast	II-III NED	KLH-MUC-1 + QS-21 id	Anti-KLH Ab but minimal anti-MUC-1 Ab Inconsistent CTL response	None reported

STn: sialyl Tn; KLH: keyhole limpet hemocyanin; id: intradermal; iv: intravenous; PR: partial response; BCG: bacille Calmette Guérin; Bx: biopsy; CTL: cytotoxic T lymphocytes; CTX: cyclophosphamide; NED: no evidence of disease; Ab: antibody.

15 patients and CTL responses were observed in 2 of 10 tested patients as measured by chromium release assay after two cycles of stimulation. Toxicity was minimal and consisted mainly of erythema and pruritis at the injection sites. Clinical responses were reported separately and combined with data from two other phase I trials evaluating the same vaccine strategy in other patients with adenocarcinomas (45). Of the 41 patients in this combined report, 5 had stable disease (3 of which had breast cancer) and the rest had disease progression.

Snijdewint et al. vaccinated 10 stage I–IV breast cancer patients with 33– and 106–amino acid MUC-1 repeats conjugated with KLH (46). Both the 33– and the 106–amino acid peptides induced antibody titers (up to a 10-fold increase) compared with prevaccinated plasma in all treated patients. CTL activity was measured in vitro by chromium release assay and was also increased in the post-vaccination patient samples.

In a recent report by Musseli et al. the validity of current measurements off MUC-1-specific T-cell responses was questioned (47). In this study, a 106–amino acid MUC-1 peptide was conjugated with KLH plus QS-21 to vaccinate stage II–III breast cancer patients. Six patients underwent leukapheresis for repetitive immune monitoring. ELISPOT and T-cell proliferation assays were performed with and without IL-2 after 20-h or 6-d stimulations. All patients generated anti-KLH, and anti-MUC-1 antibodies. All patients also demonstrated consistent generation of T-cell responses against KLH by both ELISPOT and T-cell proliferation assays. In stark contrast, anti-MUC-1 T-cell responses were weak and inconsistent with the ELISPOT and T-cell proliferation assays often contradictory. The intrapatient and interassay variability observed in this study suggests convincing evidence of anti-MUC-1 cell activity is still lacking. These results may also explain the frequently observed discordance between immunologic immune responses and clinical responses. As few investigators use repetitive and/or redundant immune monitoring in their clinical trials, the immune response data may be unreliable and thus not predictive of clinical responses.

Carbohydrate epitopes, as well as peptide components of glycoproteins and mucins, may be immunogenic. Thus, carbohydrate-derived antigens that are preferentially expressed on malignant ovarian and breast tissue are also attractive vaccine targets. These include the O-linked mucin glycans (Tn, T, TF, and STn). Elevated serum STn has also been shown to be an independent predictor of poor prognosis in ovarian cancer patients (48) and thus may be involved in cancer pathogenesis.

The most widely studied MUC-1-associated carbohydrate epitope vaccine is the Theratope® vaccine produced by Biomira, Inc. (Edmonton, Alberta, Canada). Theratope is administered subcutaneously and contains STn, a disaccharide antigen expressed on the core region of MUC-1, conjugated to KLH and typically administered with DETOX™-B (also known as Enhanzyn™ Immunostimulant), a lyophilized oil droplet emulsion containing monophosphoryl lipid A and cell wall skeleton from *Mycobacterium phlei*. In initial studies, patients treated with Theratope generated both cellular and humoral immune responses and a trend toward improved survival was observed. MacLean et al. vaccinated 12 breast cancer patients with minimal metastatic disease with Theratope, after a single dose of intravenous cyclophosphamide (49). All patients developed STn-specific IgM and IgG responses as measured by ELISA. Nine of 11 patients studied had significantly increased post-vaccination complement-mediated cytotoxic lysis of breast cancer cell lines expressing STn (DU 4475) as measured by chromium release assay. All patients exhibited DTH at the vaccination site. Significant toxicity was noted at the

injection site with five patients developing fluctuant granulomas. Two of the 12 patients had clinical evidence of tumor regression and 4 of 12 had stable disease for 6–10 mo after vaccination.

In a larger Theratope trial, 85 patients with metastatic breast, ovarian, and colorectal cancer were vaccinated (50,51). This study confirmed generation of anti-STn IgG and IgM responses in 83 of 85 patients. Anti-STn antibody response correlated with increased survival in both breast and colon cancer patients. This study also demonstrated that the use of one single dose of intravenous cyclophosphamide was correlated with increased survival ($p = 0.00176$), and increased anti-STn titers compared with vaccination without cyclophosphamide or with oral cyclophosphamide. The use of low-dose intravenous cyclophosphamide is thought to inhibit suppressor T cells and has been shown to increase immune responses in melanoma patients and animal studies. In a third study of 40 patients, a trend toward survival was also seen in ovarian and breast cancer patients vaccinated with Theratope following a stem-cell transplant compared to historical controls who received transplant alone (52). Those patients displaying specific anti-STn immune responses in vitro as measured by lytic activity against an STn-expressing tumor cell line tended to remain in remission longest. Eight patients had moderate to strong local DTH reactions at the vaccination sites as well. A large phase III trial in patients with metastatic breast cancer completed accrual in March 2001 with over 1000 patients randomized between vaccination with Theratope and KLH alone. The primary end point of improved overall survival was not met at the final analysis (although the results have not been published yet).

Despite the numerous trials testing MUC-1 vaccines in cancer patients, we still do not know the optimal MUC-1 peptide, adjuvant, dosing schedule, or immune monitoring for determining efficacy. Both humoral and cellular responses have been documented, but the potency of the immune response is variable and may be dependent on the type adjuvant, MUC-1 antigen administered, immune monitoring assay utilized, or HLA type of the patient. Clinical responses are rarely reported and a clear relationship with the generation of an immune response is still lacking.

4.2. HER-2/neu

For over a decade, the HER-2/neu proto-oncogene has been a target of vaccine and immunotherapies. The HER-2 protein (p185 HER-2) is a transmembrane epidermal growth factor receptor (EGFR) found in normal and malignant breast tissue, as well as ovarian, pancreatic, endometrial, non-small-cell lung cancer, and prostate cancers (53–55). The HER-2/neu proto-oncogene is located on chromosome 17 and encodes the 185-kd HER-2 tyrosine kinase receptor. HER-2/neu is overexpressed in up to 30% of breast cancers and is a poor prognostic factor in breast cancer (56–59). Trastuzumab (Herceptin), an antibody directed against HER-2/neu, has shown impressive clinical activity in HER-2/neu-overexpressing breast cancer and is approved for the treatment of patients with metastatic breast cancer (59).

Many of the HER-2 vaccine studies to date have involved peptide-based vaccines in breast and ovarian cancer patients. In these phase I trials, the generation of cellular and humoral immunity has been the primary end points and few clinical response data have been reported (see Table 6). As HER-2 is a large molecule that traverses the cell membrane, identification of the most immunogenic peptide sequences has been a major focus (60).

Table 6
HER-2/neu Vaccine Trials

Citation	Phase	N	Disease	Stage	Vaccine Approach	End Points	Toxicities
(61)	I	8	Breast	III–IV	HER-2/neu 15–18 aa peptides of ICD and ECD + GM-CSF id	8/8 peptide specific T-cell responses 6/8 with epitope spreading	None reported
(63)	I	32	Breast, ovary, lung	III–IV	HER-2/neu 13–18 aa peptide combinations, id	DTH responses correlated with T-cell responses	None reported
(62)	I	19	Breast, ovary	III–IV	HER-2/neu 15 aa peptide, HLA-A2 restricted, id	78% specific T-cell proliferation 28% w/epitope spreading	1 pt with Grade 2 skin rash
(87)	I	10	Breast, ovary	III–IV	HER-2/neu ICD peptides + Flt-3 ligand ± GM-CSF	specific CTL minimal but precursors noted	1 pt with reversible Sicca syndrome
(64)	I	64	Breast, ovary, lung	III–IV	HER-2/neu ICD or ECD peptides id	13 of 14 patients who completed therapy generated T-cell immunity	6 pts with Grade 1–2 skin rash; 1 pt with myalgia

aa: amino acid; ICD: intracellular domain; ECD: extracellular domain; id: intradermal; DTH: delayed-type hypersensitivity; CTL: cytotoxic T lymphocytes; GM-CSF: sargramostim.

In eight patients with stage III–IV HER-2-overexpressing breast and ovarian cancer, Disis et al. studied HER-2-specific cell proliferation to various peptide vaccines from the intracellular domain (ICD) versus extracellular domain (ECD) of the HER2 molecule (61). Peptide-specific T-cell responses were generated in all patients in response to both ICD and ECD vaccines. In six of eight patients, T-cell proliferation was specific to other HER-2 epitopes not included in the vaccine, suggesting that epitope spreading had occurred. DTH responses were also noted in seven of eight patients and correlated with in vitro T-cell responses. Evidence of epitope spreading was verified in a second study of 19 patients with stage III–IV HER-2/neu-overexpressing breast and ovarian cancers who were also HLA-A2 positive (62). Patients received six monthly intradermal vaccinations with three 13– to 18–amino acid peptides encompassing both HER-2-specific MHC class II helper epitopes and HLA-A2-specific binding motifs in combination with GM-CSF. The use of the two classes of epitopes was to simultaneously generate CD4 and CD8 T-cell responses. The GM-CSF was used as an adjuvant to help attract and stimulate

Table 7
CA-125 Anti-Idiotype Vaccine Trials

Citation	Phase	N	Disease	Stage	Vaccine Approach	End Points	Toxicities
(67)	I	16	Ovary	III–IV	Anti-idiotype ACA-125 sc	9/16 generated humoral and cellular responses to CA 125 Progression-free survival longer in patients with + immune responses	1/16 with abdominal pain
(66)	I	7	Ovary	IV	Anti-idiotype ACA-125 sc	No clinical responses noted Increased Th1 and Th2 cytokine levels in all patients	None reported

sc: subcutaneous; Th1: T-helper-1; Th2: T-helper-2.

antigen-presenting DCs to the vaccination site and thus encourage epitope spreading by creating a microenvironment conducive to optimal antigen presentation and T-cell stimulation. An impressive 14 of 18 patients generated T-cell proliferative responses, and 28% of patients demonstrated epitope spreading. Additional studies confirmed that DTH responses correlated with *in vitro* T-cell activity in 32 patients with HER-2-overexpressing breast, ovarian, or non-small-cell lung cancer immunized with the HER-2 peptide-based vaccines (63). In the most recent publication using this HER-2 peptide-based vaccine approach, 92% of patients (only 38 patients who completed all six scheduled vaccinations were included in the immune analysis) have developed T-cell immunity as measured by T-cell proliferation and a stimulation index >2.0, epitope spreading was observed in 84%, and 38% had persistent evidence of HER-2-specific T-cell immunity 1 yr after their last vaccination (64). The authors conclude that simultaneous stimulation of both the CD4 helper and CD8 CTLs is superior for generating long-lived specific immune responses, though the clinical efficacy of this approach in patients with active or persistent cancer is still unknown and awaits further phase II trials.

4.3. CA-125 and CA-125 Anti-Idiotype Vaccines

One of the most studied TAAs in ovarian cancer is CA-125. This glycoprotein, derived normally from coelomic epithelium, is overexpressed in almost all ovarian epithelial malignancies and occasional breast malignancies. CA-125 is an established serum marker for diagnosis and monitoring therapies in ovarian cancer. Its function is unknown, and the gene has not been cloned, although its proximity to the locus for BRCA1 suggests it may play a role in malignant transformation (65).

An antibody vaccine has been developed using an anti-idiotype CA-125 antibody (*see* Table 7). Anti-idiotype antibodies bind to the antigen-combining sites of antitumor (idiotype) antibodies, mimicking the TAA, and thus can initiate immune responses against

the TAA (66). A murine monoclonal anti-idiotype antibody, ACA-125, was developed that binds anti-CA 125 antibodies; ACA-125 is the “internal image” of CA-125. Wagner et al. subcutaneously vaccinated 16 patients with advanced ovarian cancer with ACA-125 (67). Nine of 16 patients developed anti-anti-idiotype response as measured by human antimouse antibodies (HAMAs). Peripheral blood lymphocytes from 9 of the 16 patients induced lysis of CA-125-expressing ovarian cancer cell lines. There was a trend toward improved overall survival (11.0 ± 5.6 mo vs 8.0 ± 4.2 mo) in the patients who generated immune responses. Two patients with chemoresistant, metastatic disease had prolonged disease stabilization (16–19 mo).

This same group later vaccinated seven additional patients with advanced ovarian cancer with the anti-idiotype ACA-125 vaccine (66). The generation of intracellular cytokines, IFN- γ , IL-2, IL-4, was measured in the peripheral lymphocytes in all patients after minimum of four immunizations with anti-idiotype ACA-125 vaccine. However, the duration of immune responses was transient and trended downward by Day 16 to 32 after each vaccination.

4.4. Cancer-Testis Antigens

Cancer-testis antigens derive their name as their expression is relatively restricted to testicular tissue and various malignancies. Antigens in this family include MAGE, BAGE, GAGE, NY-ESO-1, SCP-1, SSX-2, and LAGE-1. These antigens have been largely discovered after being identified as the targets of CD8+ CTLs in melanoma patients (i.e., MAGE, BAGE, and GAGE) or by the cloning technique SEREX (serological analysis of recombinant tumor cDNA expression libraries) (68). Besides testis tissue, these antigens have been identified by immunohistochemistry or RT-PCR in a host of malignancies including melanoma, breast, and gastrointestinal cancers (69). These antigens have the advantage of not being expressed in normal tissue found in females and thus breaking self-tolerance is not necessary. Thus, at least in females, antitumor immune responses directed against these antigens may be easier to generate. Early studies of vaccines utilizing cancer-testis antigens as the immunogen are now under way. Recruitment for a multicenter phase I/II vaccine trial involving intranodal delivery a DNA plasmid encoding HLA-A2-binding NY-ESO-1 peptides for patients with metastatic breast cancer is currently ongoing. MAGE-1 is overexpressed in up to 71% of serous cystadenocarcinomas and thus is another attractive target for vaccine protocols in breast and ovarian cancer patients (70).

4.5. Carbohydrate Epitopes

Tumor-associated carbohydrate antigens, often expressed as glycolipids or glycoproteins, are also potential vaccine targets. Besides the MUC-1-associated epitopes previously discussed (i.e., STn), Globo H, T-F, and the L y antigen are also being targeted in clinical trials (see Table 8). The earliest clinical trial reported the use of TF-KLH plus DETOX™ adjuvant in 10 patients with advanced ovarian carcinoma, after a single dose of low-dose cyclophosphamide (71). Nine patients developed increased anti-TF IgM, IgG and IgA antibodies. Serum from seven patients also demonstrated postimmunization complement-mediated lysis. All patients progressed though four had disease stabilization for 4–7 mo.

The L y pentasaccharide is overexpressed on ovarian cancers. Sabbatini et al. vaccinated 25 patients with advanced ovarian cancer who were clinically without disease with

Table 8
Carbohydrate-Based Vaccine Trials

Citation	Phase	N	Disease	Stage	Vaccine Approach	End Points	Toxicities
(72)	I	25	Ovary	I-IV NED	L ^y -KLH + QS-21 id	8/25 anti-L ^y IgM Ab 4/25 anti-L ^y IgG Ab 5/25 CR at 18 mo	Minimal
(39)	I	27	Breast	IV	Globo H-KLH + QS-21 id	16/27 IgM Ab	Mild skin reactions
(72)	I	67	Ovary	IV	4 trials with L ^y , KSA, MUC-1, and L ^y id	Ongoing	No auto-immunity

L^y: Lewis^y; KLH: keyhole limpet hemocyanin; id: intradermal; Ab: antibody; CR: complete response.

L^y-KLH conjugate vaccine plus QS-21 in a phase I trial (72). The intradermal vaccine was well tolerated. Eight of 25 patients demonstrated increased levels of anti-L^y IgM and 4 of 25 had elevated anti-L^y IgG in response to the vaccine. All developed strong anti-KLH antibody responses as well. Of nine patients who demonstrated complement-mediated cell lysis, five also demonstrated elevation of anti-L^y IgG or IgM antibody.

The hexasaccharide portion of the glyceramide, Globo H, is present on breast cancer cell lines (MCF-7), as well as a variety of epithelial cells. Twenty-seven breast cancer patients who were NED or had stable stage IV disease were vaccinated with Globo H, conjugated with KLH and QS-21 (41). An IgG serologic response to Globo H was minimal in all patients, but a strong IgM response was demonstrated in 16 of 27 vaccinated patients. Eight of these patients also demonstrated complement-mediated cell lysis to Globo-H-containing cell lines. Although not powered for clinical outcome, 13 patients had disease progression over the 85 to 135-wk trial.

A National Cancer Institute (NCI)-sponsored phase II study at Memorial-Sloan Kettering is currently evaluating a vaccine incorporating several antigens including GM2, Globo-H, Lewis^y, TF, Tn, STn, and 32-amino acid MUC-1 conjugated to KLH plus QS21 in patients with stage II breast cancer at high risk for disease recurrence (*see* Table 11).

5. DNA VACCINES

Numerous phase I studies using gene transfer/therapy in ovarian and breast cancer have been performed (*see* Table 9). Most published studies have focused on toxicity, tolerance, and gene transfer efficiency. In breast cancer, phase I trials have studied gene therapy in several settings: as intratumoral therapy to stimulate local antitumor immune responses (73,74), to decrease the myelosuppression associated with high-dose chemotherapy (75), to introduce suicide genes into tumor cells (76–78), and to reverse aberrant gene expression– and activity–associated malignant phenotypes (79–83). Although generally well tolerated, few clinical responses have been documented, gene transfer efficiencies have been low, and none of the trials have advanced into phase III development. Because of these disappointing results, most investigators are focusing on improving

Table 9
DNA Vaccine Trials

Citation	Phase	N	Disease	Stage	Vaccine Approach	End Points	Toxicities
(73)	I/II	10	Ovarian Cervical	IV	Intratumoral DNA/lipid complexes encoding human HLA-2, HLA-B13, or murine H-2k genes	No systemic clinical responses Local regression in 6/8 HLA-A2 vaccine	Well-tolerated
(74)	I	8	Breast	IV	Intratumoral xenogeneic fibroblasts transfected ex vivo with human IL-2	No objective responses 2/8 injection sites with increased T-cell and NK-cell infiltration	Well-tolerated

vectors, novel delivery systems, and combining gene transfer techniques with vaccine strategies, i.e., genetically modified DC vaccines.

A common strategy of DNA vaccines is to nonspecifically stimulate T and B cells in the tumor microenvironment to improve host immunocompetency in recognizing TAAs released or presented in the area. To this end, DNA plasmids encoding allogeneic HLA antigens and/or stimulatory cytokines have been injected intratumorally. Hui et al. treated 10 patients with extensive ovarian or cervical cancer with intratumoral injections of DNA-encoding human HLA-A2, HLA-B13, and murine H-2k genes complexed to cationic lipids (73). All patients developed systemic disease progression, though local regression at the injection site was noted in six of eight patients who received the HLA-A2 gene. All of these patients were HLA-A2 negative and thus presumably were developing an allogeneic response at the injection site that accounted for the tumor regressions. As systemic responses were not seen, it is unclear how useful transferring allogeneic genes will be in generating specific antitumor immune response against TAAs. This approach in other tumor types, particularly metastatic melanoma, has been disappointing to date (84). Tartour et al. used a similar technique of provoking an intense immune response, in this case against an xenogeneic cell, by injecting monkey fibroblasts that had been genetically modified to secrete human IL-2 intratumorally in eight breast cancer patients (74). Although well tolerated, no clinical responses were noted and exogenous mRNA IL-2 was not detected within the injection sites. There was histologic evidence of immune activation in two of eight injection sites as determined by natural killer (NK)- and T-cell infiltration, most likely in response to the xenogeneic cells.

6. DENDRITIC CELL VACCINES

DCs are potent antigen presenting cells (APCs) capable of initiating primary immune responses (85). Vaccine strategies designed to exploit the APC activity of DCs include

Table 10
Dendritic Cell Vaccines

Citation	Phase	N	Disease	Stage	Vaccine Approach	End Points	Toxicities
(85)	I	10	Ovary, breast	IV	HLA-A2-restricted DCs pulsed with MUC-1 or HER-2/neu intranodal	5/10 with peptide-specific CTL 2/10 with epitope spreading 1 clinical response	None reported
(87)	I	10	Ovary, breast	IV	Flt-3 ligand coadministered with HER-2/neu peptide vaccine	T-cell proliferation not observed DTH seen in 3/4 patients who received GM-CSF No clinical response data	None reported
(88)	I	8	Ovary, uterine	IV	DCs pulsed with KLH and tumor lysates intracutaneously	3/6 stable disease up to 7 mo 6/8 T-cell immunity to KLH 2/8 T-cell immunity to lysate	Cutaneous hypersensitivity

DC: dendritic cell; CTL: cytotoxic T lymphocyte; DTH: delayed-type hypersensitivity; GM-CSF: sargramostim.

leukapheresis of patients to obtain large numbers of autologous DCs, isolation and expansion of autologous DCs in vitro, activation of the DCs against TAAs by antigen/peptide loading or gene transfer, and then reinfusion of the DCs subcutaneously, intra-dermally, intralymphatically, or intravenously to induce antitumor immune responses. Other strategies include administering cytokines, especially GM-CSF and Flt-3 ligand, in combination with peptides to recruit DCs to the vaccination site. Several ongoing vaccine trials are testing the use of DCs in generating specific antitumor immune responses in patients with breast and ovarian cancer (Table 10).

In an early trial, Brossart et al. reported the results of 10 patients with metastatic breast or ovarian cancer treated with at least three subcutaneous injections of autologous DCs pulsed with HER-2/neu or MUC-1 peptides (86). Three ovarian and one breast cancer patient received the MUC-1-pulsed DCs, whereas five breast and one ovarian cancer patient received the HER-2/neu-pulsed DCs. The vaccinations were well tolerated. One breast cancer patient who received MUC-1-pulsed DCs had a partial clinical response with regression of visceral disease at 4 mo, but development of brain metastasis at 5 mo. One other patient had disease stabilization for 8-mo duration. Five patients developed specific T-cell responses as measured by intracellular cytokine production. Peptide-specific CTLs, as measured by chromium release assays, were also observed in

Table 11
Current Vaccine Trials Listed on NCI Clinical Trial Website

Sponsor	Phase	Anticipated Accrual	Disease	Stage	Vaccine Approach
Duke University, NCI	I	6	Gastric, breast, ovarian cancer	II-IV	Autologous DCs pulsed with HER-2/neu intracellular domain, followed by autologous DCs pulsed with tetanus toxoid and KLH
Fox Chase Cancer Center, NCI	I	48	Ovarian, breast, gastric, lung, head & neck, testicular, primary liver, colorectal cancer	III-IV	Recombinant viral vaccine, fowlpox—CEA with or without sargramostim (GM-CSF)
NCI	I	45	Ovarian	III-IV	Wild-type p53 peptides admixed with GM-CSF or pulsed on DCs with low-dose IL-2 and ISA-51 in HLA-A2-positive patients
NCI	I	45	Breast	IV	Wild-type p53 peptides admixed with GM-CSF or pulsed on DCs with low-dose IL-2 and ISA-51 in HLA-A2-positive patients
Southwest Oncology Group, NCI	I	20	Ovarian, prostate, lung, gastric cancer, brain tumor	II-IV	Randomized, epithelial growth factor receptor peptide vaccine with GM-CSF or KLH
NCI	II	112	Breast, colorectal, melanoma, non-small cell lung, sarcoma	III-IV	MAGE-12 peptide and Montanide ISA-51 (as adjuvant)— <i>completed accrual</i>
NCI	I	70	Breast, colorectal, gyn, lung, and pancreatic cancer	III-IV	Autologous PBMC pulsed with p53 or ras peptides, + GCSF
University of Connecticut, NCI	I/II	15	Breast	IV	Autologous purified heat shock proteins as consolidation after standard therapy for stage IV disease
NCI	II	28	Breast, high risk	II-III	Following treatment with adriamycin, cyclophosphamide, and paclitaxel, vaccination with recombinant Vaccinia-CEA-TRICOM
NCI	II	62	Breast	IV	Following treatment with adriamycin, cyclophosphamide, and paclitaxel, vaccination with recombinant Vaccinia-CEA-TRICOM
Memorial Sloan- Kettering Cancer Center, NCI	I	30	Breast	I-IV	Vaccination with multiple antigen conjugate: Globo-H-GM2-Lewis ^y - MUC1-32(aa)-STn(c)-TF(c)-KLH conjugate with QS-21
St. Vincent Medical Center, NCI	II	20– 40	Breast, pancreatic, lung, brain, colon, melanoma, kidney	III-IV	After intravenous cyclophosphamide, autologous or allogenic tumor cell
Biomera, Inc.	II		Breast	IV	Theratope® vaccine with concurrent hormonal suppression

NCI: National Cancer Institute; DC: dendritic cell; GM-CSF: sargramostim; KLH: keyhole limpet hemocyanin; IL-2: interleukin-2; PBMC: peripheral blood mononuclear cell; GCSF: filgrastim.

the five patients who exhibited increased intracellular IFN- γ levels by flow cytometry. Interestingly, there was evidence of CTL activity against cell lines not expressing the vaccinated antigen. One patient who received the HER-2/neu-pulsed DCs developed CTLs against MUC-1, but not HER-2-expressing cell lines. An optimistic interpretation of this result would be that DC vaccines are capable of inducing primary antitumor immune responses through epitope spreading.

The use of Flt-3 ligand, a cytokine that is a potent DC stimulator, can increase circulating DCs up to 40-fold (87). Consequently, groups have looked at infusing Flt-3 ligand as a strategy to avoid the time and costs associated with ex vivo DC generation. Flt-3 ligand has been used as an adjuvant in a HER-2/neu peptide-based vaccine trial by Disis et al. (87). Ten patients with either stage III or IV breast or ovarian cancer whose tumors overexpressed HER-2/neu were administered Flt-3 ligand concurrently with an HER-2/neu ICD peptide vaccine with or without GM-CSF. Post-vaccination DTH responses were noted in three of four patients who received Flt-3 ligand plus GM-CSF, but HER-2/neu-specific T-cell responses as measured by ELISPOT were not observed in any patient. Clinical response data were not presented.

In a small phase I trial involving patients with advanced ovarian or uterine cancer, eight patients received DCs pulsed with KLH and autologous tumor lysates (88). The vaccine was well tolerated and three patients (all with ovarian cancer) had disease stabilization lasting 25–45 wk.

Though few vaccine trials involving DCs have been published, many additional DC trials are currently in progress testing various TAAs, DC isolation methods, routes of administration, and adjuvants (*see Table 11*). The optimal method for antigen delivery to DCs is another avenue of active research. Direct peptide or tumor lysate loading onto MHC, gene transfer to allow the protein to be intracellularly processed by the DC prior to MHC presentation, and DC-tumor cell fusions are being tested in clinical trials designed to determine the optimal approach for expressing tumor antigens on DCs and generating cellular immunity.

7. CONCLUSIONS

Recent advances in understanding the endogenous immune response to breast and ovarian cancers have spearheaded the current interest in immune therapy for the treatment of these cancers. Thus far, several vaccine trials in patients with high-risk stage II or advanced-stage cancers have been completed. Although these small early trials support the safety of vaccine therapy, end points have been primarily immunologic, and not clinical antitumor response. Despite numerous trials focusing on immune monitoring, the ideal method for measuring *in vivo* immune responses, or even whether these responses will correctly correlate with clinical antitumor activity is still unknown. Methodologies are dependent on the investigational group leading the trial, often poorly described in the published manuscript, and technically difficult and expensive to perform. Clinical trials are often too small for proving any results beyond safety and feasibility and published only in abstract form. Thus, no clinical trial has definitely established a route, schedule, adjuvant, antigen, and/or dose for effective vaccination.

Clinical trials support that vaccine therapies are well tolerated and maximum tolerated doses (MTDs) have not been reached. The only consistent toxicity is local inflammatory

reactions at administration sites. Despite the potential for significant autoimmune toxicity, as vaccines are often directed against TAAs that are also expressed in normal organs, this has not been a problem to date. This may simply be a reflection of the inability of current cancer vaccines to break self-tolerance and generate long-lasting specific immune responses against any, including tumor-associated, self-antigens.

The majority of phase I studies published have been performed on small cohorts of patients with advanced disease. Cancer vaccines are most likely to be effective in patients with low tumor burden and intact immune systems. Patients who are heavily pretreated are unlikely to generate immune responses capable of overcoming self-tolerance and eliminating large tumor burdens. The safety profile of vaccines is sufficient to move clinical trials to patients with small or unmeasurable tumor burdens such that other mechanisms of immune escape can be studied. Homing of tumor-reactive CTLs to the tumor sites, immunoinhibitory cytokines released by the tumor cells, and downregulation of immune recognition molecules by the tumor cells are a few of the established mechanisms by which tumor cells escape immune surveillance that will need to be overcome if vaccines are to be successful. Generation of both humoral and cellular antitumor immune responses will probably be necessary for effective therapy. Multiple antigens or peptides may be needed to mobilize both helper as well as CTL responses and avoid immune escape through downregulation of the targeted antigen. Larger clinical trials will require more resources as well as standardization of immune monitoring and vaccine strategies such that multicenter trials will be possible.

The hope of patients for a breast and ovarian cancer vaccine is strong and likely to remain so for the foreseeable future. Breast and ovarian cancer advocacy groups will likely continue to support future vaccine research and clinical trials (*see* Table 11). Thus, it is paramount for clinicians and scientists, immunologists and oncologists, to work together to design intelligent clinical trials that answer basic questions, exploit advances in our understanding of the immune system, and ultimately lead to the initiation of successful phase III clinical trials.

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31

Vaccine Therapy for Gastrointestinal Cancers

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1. INTRODUCTION

Immune therapies for gastrointestinal (GI) cancers have actually been tested over several decades. Early attempts at immunotherapy focused mostly on the enhancement of the immune response systemically and nonspecifically by the addition of agents such as interferon (IFN), interleukin-2 (IL-2), levamisole, and bacille Calmette-Guérin (BCG) (1). These efforts have not resulted in effective therapies. The more recent work has focused on enhancing the immune system against specific tumor-associated antigen(s) (TAAs). Many vaccine and immune-based therapies are/have been tested that utilize a cancer-specific approach instead of nonspecific enhancement of the immune system. These approaches are attempting to take advantage of our increased understanding of what distinguishes cancer cells from normal cells with the goal of developing effective and nontoxic immune therapies for cancer patients. It will be these therapies on which this chapter will focus.

GI cancers offer an ideal setting in which to explore vaccine therapy. First, they are common diseases making GI-specific vaccine development an important public health

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issue. Second, there is an urgent need for more effective agents for the diseases as they represent a group of disease with a terrible prognosis. Third, there are many clinical situations where immune-based therapy would be logical (adjuvant therapy for stage II/III disease, postoperative therapy following resection of metastasis, combinations with chemotherapy for advanced disease). Fourth, GI cancers express several tumor-specific antigens that have been well characterized and have high potential for immune targeting. In this chapter, we will review the current experience with immune therapies in GI cancer focusing on the clinical experience with active specific immunotherapy, recombinant gene/virus therapy, monoclonal antibody therapy, anti-idiotype therapy, and peptide-based therapy.

2. ACTIVE SPECIFIC IMMUNOTHERAPY

Active nonspecific immunotherapy, utilizing agents such as BCG, IL-2, levamisole, and diphtheria toxoid, has been used to stimulate a systemic or generalized immune response not directed against any particular tumor antigen (2). Nonspecific immunotherapy relies on the existence of immune effector cells (T and B cells) that have already recognized and processed the tumor as foreign. Then, by enhancing the overall immune response, the hope is to enhance the tumor-specific elements to create an effective anti-cancer response. Poor, nonreproducible results have led clinical researchers to move toward active specific immunotherapy (ASI) approaches. In this approach, the goal is to immunize the patient against his/her own cancer specifically, via induction of a tumor-specific immune response able to kill tumor cells (3).

2.1. *Whole-Cell ASI*

The most common and widely tested vaccine technique is ASI. ASI is vaccination with whole-tumor-cell preparations (allogenic or autologous), which are often combined with systemic immune modulators such as BCG, admixed with virus such as Newcastle disease virus (4), or even genetically altered to secrete cytokines. Whole-cell preparations possess several distinct qualities compared to other more targeted modalities of ASI. First, whole-cell preparations provide all possible tumor antigens together, including cell-surface and intracellular antigens. Second, this method affords a high tumor-antigen vs self-antigen ratio. Whole-cell allogenic vaccines are created utilizing cells of multiple patients and multiple tumor cell lines, with the advantage of enhanced immunogenicity via exposure of the patient to a wider variety of TAAs, as well as greater probability for developing standardization, thus making multiple-patient therapy a reasonable goal. However, the allogenic vaccine approach has not been the focus of more recent trials.

Whole-cell autologous vaccine preparations require a fresh sample of the patient's own tumor, either primary or derived from an established tissue culture. Primary tumor is preferred since cell lines modify the phenotype of the tumor during culture, and such cell lines remain technically difficult to grow (5). Autologous whole-cell vaccines are now being pursued in many trials, some of which are focused on GI cancers. In theory, this approach is not restricted to any particular tumor type; instead it is restricted to only those tumors that can be made into vaccines. One major issue recently uncovered is that the immune system seems to identify only a subset of peptides from within the entire tumor-specific proteins. Whole-cell approaches would have the advantage of exposing the

patients to the entire selection of peptides including the key peptides, but has as the disadvantage of exposing patients to only low doses of each peptide. In either case, the inherent heterogeneity of tumors presents the additional problem of possible underdosing of TAAs within the utilized tumor cells.

2.1.1. CLINICAL TRIALS

ASI using whole-cell autologous tumor vaccine was first successfully demonstrated preclinically in a guinea pig hepatocellular tumor model, in which dissociated tumor cells were admixed with BCG and utilized as an intradermal vaccine to stimulate systemic tumor immunity. This led to a prospectively randomized controlled clinical trial of ASI for colorectal carcinoma in which 74 postsurgical patients received two weekly intradermal injections of autologous irradiated tumor cells admixed with BCG, followed by a third injection of tumor cells alone. Results indicated a significant delay in both death and recurrence (56-mo median follow-up time) for colon and rectal cancer patients combined (6,7). Hoover et al. in 1993 demonstrated no statistically significant difference regarding survival or disease-free survival in a 6.5-yr median follow-up of patients with Dukes' stage B2-C3 colon or rectal cancer treated with resection and autologous tumor cell-BCG ASI versus resection alone. However, a subsequent cohort analysis did demonstrate statistically significant improvement in both end points mentioned above within the colon cancer ASI group (8). This study was soon followed by an Eastern Cooperative Oncology Group (ECOG) trial that examined survival and relapse rates with autologous tumor cell-BCG ASI versus surgery alone in the setting of Dukes' B and C colon cancer. Results between these groups were not statistically significant, yet a subset analysis indicated further investigation with a heightened vaccination schedule was warranted (9).

Ockert et al. utilized autologous colon cancer cells infected with Newcastle Disease Virus vs admixture with BCG, and found statistically significant improved survival at 2 yr for the viral ASI group (98% vs 67%) (4). Most recently, Vemorken et al. explored use of an additional booster immunization to the previous model of adjuvant BCG ASI, in the setting of surgically resected stage II and III colon cancer, with findings at the 5.3-yr median follow-up consistent with statistically significant reduction in the rate of tumor recurrences. Further analysis by stage failed to demonstrate any significant benefit of ASI in patients with stage III disease, however stage II patients had significantly improved recurrence-free interval, recurrence risk reduction, recurrence-free survival, as well as a trend toward prolonged overall survival (10).

This body of work testing ASI in colon cancer is often overlooked. First, this work is important for the positive results obtained, even if the most benefit was seen only in subset analyses. The techniques used in these trials were not up to today's standards and certainly our improved understanding of the immune system should make us better able to deliver more effective ASI-type therapies. Second, the investigators performed these trials in stage II and III patients, arguably the ideal group of patients in which to test vaccine approaches. There have been very few adjuvant vaccine trials in the past decade, and these investigators must be applauded for completing these difficult trials in this patient population. Third, this work has spawned our current efforts at improving ASI techniques using fusion products of tumor cells and antigen-presenting cells (APCs).

2.2. Targeted ASI

The development of TAA-directed vaccine involves identification of a target antigen and selection of a platform (e.g., proteins, peptides, carbohydrates, and DNA- or viral-based vectors) for presentation to the immune system. Potential target candidates are antigens that are expressed only on tumor cells or only those that are relatively overexpressed on tumor cells compared to normal cells. Very few antigens are truly tumor-specific to a particular patient's tumor. Some may be limited in expression to a particular tumor type whereas others have a wide expression on a variety of different tumor types. Recently molecular techniques such as SERAX analysis, microarrays, SAGE, and differential displays are being used to identify new antigen targets for vaccine targeting (11).

GI cancers are good models in which to examine targeted ASI, as they have well-characterized and most likely a finite number of TAAs, such as carcinoembryonic antigen (CEA) and 17-1A (12). However, these questions remain: are these available agents truly the optimal ones for clinical testing, and which is the most optimal? The remainder of the chapter will focus on further exploration of the following targeted ASIs within the setting of colorectal cancer: recombinant viral vector-encoding TAAs (vaccinia-CEA, ALVAC-CEA); anti-idiotype antibodies that mimic TAAs (CEAVac, 17-1A); monoclonal antibodies (17-1A/Panorex, L6); glycoprotein mucins (MUC-1); peptides encoded by oncogenes (K-ras, P53); and dendritic cells.

3. RECOMBINANT VIRAL VACCINES

As a means to increase antigen processing and expression in APCs and improve the expression of costimulatory molecules, recombinant viral-based vaccines have been developed. The pox virus family has been most commonly used (vaccinia, fowlpox) but others such as adenovirus have been used. Genes encoding TAAs are genetically recombined to the virus and then administered. The virus serves as a vector, infecting cells including APCs; the passenger gene is then transcribed and translated into a full-length protein, then cleaved into smaller peptides (9–10 amino acids in length for major histocompatibility complex [MHC] class I, 13–15 amino acids for MHC class II presentation) and then presented on the cell surface in the context of appropriate costimulatory molecules to activate both CD4 and CD8 T cells (13).

Phase I clinical trials have been completed for both vaccinia-CEA (14,15) and ALVAC-CEA (16). These trials both demonstrated significant generation of CEA-specific cytotoxic T lymphocytes (CTLs) that were capable of lysing autologous (and allogeneic) tumor. The vaccines were well tolerated without significant toxicity. Vaccinia was found to be a potent stimulus upon initial vaccination but subsequent injections added little. It is important to note that all patients on this trial had received prior vaccinia vaccinations, which may of course have influenced the outcomes. Fowlpox, on the other hand, was less potent but immune responses measured as CEA-specific T cells increased with each injection. This suggests that fowlpox-CEA could serve as an effective booster to the immune response.

Based on this clinical experience and strong preclinical data (17,18), a trial combining vaccinia-CEA (V) and fowlpox-CEA (F) was performed. In the first stage of this trial, patients with CEA-bearing cancers were randomly assigned to receive either V-F-F-F or

F-F-F-V (19). Both safety and immune response were end points. The vaccines were very safe with virtually no toxicity observed that was attributable to the vaccines. In addition, the immune response data as measured using ELISPOT analyses of CAP-1 T cells demonstrated a significantly greater response for patients receiving V-F-F-F as compared to the other sequence. Supporting the clinical relevance of this immune data was the observation that the V-F-F-F group had a significantly longer survival than those randomized to the other arm. Therefore, the clinical data corroborated the preclinical data suggesting that the “prime and boost” schedule was superior to one vaccine alone. This conclusion remains controversial and further studies are required to confirm this finding. Though recent global events have made us less concerned about using vaccinia as a viral vector for vaccines, we must recognize that there is a known life-threatening toxicity associated with vaccinia. Therefore, we must be certain of its importance as we go forward.

Several clinical trials have followed on the above prime-and-boost trial. The first was the serial addition of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-2 along with the vaccines (20). Presented in abstract form, the results showed that GM-CSF did generate a greater T-cell response compared to vaccines alone and clinical benefit was seen in several patients including a complete tumor response. However these patients were not randomized. The IL-2 group did not show any further advantage over the GM-CSF group and did experience a higher degree of toxicity associated with the IL-2.

3.1. Costimulatory Molecules

The above pox viral vector vaccines used only the gene for CEA alone. An increased recognition of the role of costimulatory molecules has sparked an interest in modifying the CEA vaccines to also include the genes for one or more costimulatory molecules. The first constructs combined the pox viral vectors with CEA and B-7. The trials testing this agent alone and in combination with GM-CSF demonstrated a high level of patient safety and significant T-cell responses, including the demonstration of T-cell infiltrations into the skin. Less clear was the role of GM-CSF and the relative contribution of the B-7 gene addition (21). Currently, there is a phase II trial testing the fowlpox-CEA-B7 vaccine in combination with chemotherapy in patients with untreated metastatic colon cancer. This trial is designed to answer several key questions about the vaccines and chemotherapy.

Other vaccine constructs have now been tested. We have just completed a phase I trial of vaccinia-CEA(6D)-B7-ICAM1-LFA-3 (vaccinia TRICOM) and fowlpox-CEA(6D)-B7-ICAM1-LFA3 (fowlpox TRICOM) (22). These constructs contain the full-length gene for CEA with a point mutation in the CAP-1 peptide sequence that enhances the APC presentation to the T cell. They also contain the genes for three costimulatory molecules B7, ICAM 1, and LFA-3. The trial established the safety of fowlpox TRICOM alone, in combination with vaccinia-TRICOM, and with GM-CSF. Significant immune responses and clinical benefit has been observed in this trial. Plans to take these constructs into larger trials to address the role of concomitant chemotherapy and cytokines are under way.

4. MUC-1

Clearly CEA is a logical target for GI malignancies, but several others are being tested in similar viral vector constructs. The most tested of these is the MUC-1 antigen. Glycoprotein mucins play a large role in normal cells of the GI tract. The observation that

mucins from tumor cells are distinct with short sugar side chain suggested a potential immunologic target. Mucin 1 (MUC-1) was found to be ubiquitously expressed in breast cancer and highly immunogenic in mice. CTLs have been isolated from patients' lymph nodes that were capable of lysing tumors in an unrestricted MHC/MUC-1 system (23).

Based on this strong preclinical data, a phase I study was performed in patients with advanced colon, rectum, breast, and stomach cancers using MUC-1 fusion protein (24). The results showed large amounts of immunoglobulin (Ig)G1 anti-MUC-1 antibodies were produced in 13 of 25 patients, T-cell proliferation was found in 4 of 15 patients, and CTL responses in 2 of 10 patients. Therefore, this MUC-1-based construct was found to be highly immunogenic in cancer patients with a dose response observed for antibody production (increased dose led to higher antibody titers). Previous studies using MUC-1 had demonstrated a relationship between antibody levels and survival in patients, suggesting a need to study these compounds further in larger trials (25). A phase II study using a MUC-1 peptide admixed with BCG was explored in 30 patients with advanced colon cancer. The observed systemic symptoms and injection site reactions suggested an activation of the immune system and 7 of 22 patients tested demonstrated a two- to four-fold increase in CTLs (26).

Clinical trials using MUC-1 alone and in combination with B-7 and dendritic fusions are now under way. Preclinical data suggest an advantage to MUC-1 and B7 using the vaccinia construct (27). Tanaka et al. have demonstrated the feasibility of MUC-1 dendritic cell fusions and the reversal of immunologic unresponsiveness in mouse models and have started a clinical program with these concepts (28). Plans are also under way to combine the CEA vaccines and the MUC-1 vaccines in the hopes of generating a greater clinical benefit by targeting two established tumor antigens at the same time. This approach may help to overcome the relative high level of tumor resistance observed during the clinical trials performed to date. In these trials, a very high percentage of patients develop T-cell immune responses, but the majority of patients fail to demonstrate clinical benefit. This frustrating observation may be overcome by multitargeted strategies. In comparison to the T-cell responses seen with naked DNA, recombinant viral therapies appear superior at this stage in development. This is likely due to the increased role of the APCs, the environment created during the "infection" with the virus, and possibly avoiding triggering self-recognition often observed with naked-DNA therapies. Using cytokines, costimulatory molecules, and multiple antigens may further increase the activity of these constructs. Potential problems with this approach include lower specificity of binding of the virus to the target APCs, potential downregulation of class I molecules impairing antigen presentation, induction of antiviral responses that limit subsequent immunizations, and the potential dangers of administering viruses (even though attenuated) to humans.

Other similar approaches include the insertion of an immunostimulatory cytokine (tumor necrosis factor [TNF], IL-2, GM-CSF, IFN- γ) instead of the TAA, creating cells that secrete cytokines, and similarly creating a cell that overexpresses a native or mutated form of the TAA to improve antigen processing. These agents have not been tested in GI-specific populations and therefore will not be elaborated here.

5. β -HCG AS A TARGET

β -HCG represents an interesting target for immune therapies for GI cancers (29). The protein is expressed on the majority of colon and pancreas cancers and not on normal tissues apart from female reproductive organs. Several groups have developed vaccines to take advantage of this target. CTP37-DT is a conjugate of the C-terminal peptide of β -HCG and diphtheria toxoid and has been extensively tested in a series of clinical trials for GI cancers. Following phase I trials (30,31), two phase II trials have been completed. The first was a trial of 77 patients with metastatic colon cancer who were randomly assigned to one of two doses of vaccine. The vaccines were well tolerated and anti-HCG antibodies were induced in most patients. Interestingly, there was an association between antibody response and survival (32). The second study was a phase II randomized trial of vaccine alone compared to vaccine plus gemcitabine in 55 patients with untreated metastatic pancreas cancer. The results demonstrated that gemcitabine had little influence on whether patients had an antibody response but it did negatively alter the titer. The group receiving both vaccine and gemcitabine had a longer median survival (6.6 mo vs 4.7 mo) (33). Given the lack of toxicity, this combination will soon be tested in a phase III trial.

6. GASTIN AS A TARGET

An immune-based therapeutic called G17DT works by way of an antigastrin immunogen that produces neutralizing antibodies directed against gastrin-17. Therapeutic efficacy of both passive and active therapy with G17DT has been demonstrated in GI cancers and additive effects are seen when coadministered with 5-FU (34). At present, several trials have been completed or are under way using this compound in colon and pancreas cancers, including a phase III trial in pancreas cancer. Phase II data demonstrated that pancreas cancer patients could mount an adequate antibody response to G17DT and was well tolerated. Significantly, as with the β -HCG trial, patients who developed a higher antibody response experienced a longer survival (35).

7. ANTI-IDIOTYPE

When a monoclonal antibody is administered, it can serve as an antigen itself with subsequent generation of an antibody directed against the idiotype. This process, called the idiotype network, was originally hypothesized by Linderman and Jerne (36), and is now the subject of significant basic and clinical research. Several anti-idiotype antibodies are being tested in clinical trials against colon cancer. The lead compounds target CEA, 17-1A.

The generation of anti-idiotype antibodies begins with the immunization of an animal with the TAA of choice (e.g., CEA) and the subsequent production of an antibody against the TAA (named Ab1). Ab1 is then used to generate a series of anti-idiotype antibodies called Ab2. Selected Ab2 antibodies effectively mimic the three-dimensional structure of the original TAA, which can then be used as a surrogate for the TAA as an immunogen, with the final result of generating anti-anti-idiotype antibodies. Although this may seem like a long way to go to get back to the original antigen, there are significant advantages to this strategy. The primary advantage is that the anti-idiotype represents an exogenous

protein that expresses the target antigen whereas the original antigen itself (e.g., CEA) is a native (self) protein. The native antigen has thus been converted to a foreign protein that will be endocytosed by APCs, processed, and presented to T cells to activate the immunologic activities of CD4 and CD8 T cells. Through this, the production of endogenous cytokines increases, further stimulating the response (37). This would not be expected from simply vaccinating with the TAA itself.

Foon et al. have studied an anti-idiotype antibody directed against CEA called CEAVac. Using this compound in patients with various stages of colon cancer, they have demonstrated that the patients generate high polyclonal anti-CEA responses and idiotypic-specific T-cell response, 75% of which were CEA specific. Several patients received 5FU-based chemotherapy during the vaccinations and responses were not adversely affected. Finally, they have demonstrated the ability to boost the antibody response with monthly injections of CEAVac. Though nothing definitive can be said about these patients' clinical response given the variability in stage and low numbers, seven of eight patients with resected stage IV disease all remain on study without evidence of recurrence (12–33 mo) (38).

In a related study, 32 patients with advanced colon cancer were treated with 3H1 (TriAb), an antibody that targets human milk-fat globule (HMFG). All patients generated antibody and CD4 responses; 75% were CEA specific (39). Currently, the TriAb and the CEA Vac are being tested together in a phase II trial under CALGB. The patients selected for this trial are stage IV colon cancer patients with isolated liver metastasis that have been removed. The results of this trial are eagerly awaited and accrual must be completed soon given newer chemotherapy treatments for colon cancer now available.

The anti-idiotypic monoclonal antibody 105AD7 mimics the TAA 791Tgp72 (CD55), which is expressed on 70–80% of colorectal cancers (40). CD55 plays a role in signaling between the innate and adaptive immune responses. Absence of the molecule makes the tumor cell susceptible to complement, whereas overexpression results in the antigen being a target for T-cell immunotherapy. A phase I trial in advanced-colorectal cancer patients showed the anti-idiotype vaccine to be nontoxic with a suggestion of improved survival among vaccinated patients compared to historical paired controls (41). This effect is thought to be mediated by antitumor T-cell responses. To test this theory, 19 patients with colorectal cancer were immunized prior to their resection of the primary tumor. Tumor samples were found to have a significantly higher activated lymphocyte infiltration compared to nonvaccinated patients. Further analysis of the infiltrating cells showed them to contain a high level of both natural killer (NK) and CD4 cells, and evidence of CD8 activation (42). This evidence supports further study with this vaccine in colorectal cancer patients and also demonstrates the value of translational studies in early clinical trials.

7.1. 17-1A

The most extensively studied compound is 17-1A, a murine IgG2a MAb that targets a 26 Kda polypeptide tumor-associated cell-surface glycoprotein, GA733-2. Although this agent may function as a monoclonal antibody mediating complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity (ADCC), and apoptosis, it also may induce anti-idiotypic immune responses. As a single agent in advanced colon cancer, a 5% response rate was observed with very little toxicity. It has also been tested in

combination with GM-CSF (43). This agent was tested as adjuvant therapy in patients with stage III colon cancer in a small (189 patients) randomized trial comparing 5 mo of 17-1A injections to observation alone (44).

The primary end points of the trial were overall and disease-free survival. The results were initially presented after 5 yr and re-presented after 7 yr, and continue to show a 32% reduced mortality and 23% reduced recurrence rate in the treatment arm. The reduction in metastatic (distant sites) was significant while local recurrences were not altered between the two arms, but this latter difference could be explained by 11 patients in the control arm receiving radiation with none of the treatment arm receiving it. It also could be explained by some unknown difference in the biology of local and metastatic recurrences. Eighty percent of patients developed human antimouse antibodies (HAMA) after the second or third infusion.

Regardless, these data were compelling enough for the agent to gain approval in Germany and launch two large randomized trials, 5FU/LV ± 17-1A in stage III and 17-1A vs observation in stage II colon cancer. The first of these trials has now been presented and the results are disappointing. Although there was an improvement in survival for patients treated with the vaccine and chemotherapy compared to chemotherapy alone, the Food and Drug Administration (FDA) did not agree with that conclusion and further development of this agent is not planned (45). The second trial of vaccine compared to no therapy in stage II colon patients has completed accrual but the results are not yet available. Interest in this compound has lessened after the publication of the first trial but would be quickly rekindled if the second one is positive. One issue that has been raised following the first trial is the possibility that the chemotherapy may have interfered with the immune response to the antibody, again pointing to the importance of understanding the relationship of chemotherapy and immune therapies.

8. PEPTIDE VACCINES

Tumors express peptides, often oncogenes, that are unique to the malignant cells. Typically, these proteins are mutated forms of the native protein. Though variation in the specific mutations seen would make immunologic targeting difficult across a patient population, several oncogenes share significant homology among patients. For example, K-ras commonly is mutated in codon 12 and could serve as a potential target for vaccine development.

Several investigators are testing this hypothesis in patients. The methods include obtaining an individual patient's tumor and determining the presence or absence of particular oncogene mutation. If present, patients are then treated with a vaccine that targets the mutated section of the protein. The obvious advantage of this approach is that because normal cells do not express the mutated gene, very little systemic toxicity would be expected. However, as with all molecularly targeted therapies, tumor heterogeneity could result in escape for those clones without the specific mutation. Ras and P53 vaccines are currently under clinical investigation (46). Using a 13-mer mutated Ras peptide reflecting the codon 12 mutations, 3 of 10 evaluable patients generated a mutant Ras CD4+ and/or CD8+ T-cell response. No toxicity was observed. The current trial involves a similar strategy but adds IL-2 and GM-CSF. A recent report from this group of investigators tests this treatment in a phase II trial of resected stage III and IV colon and pancreas cancer patients. In the first 11 patients, only 3 had progressed at the time of the

writing and minimal toxicity was observed (47). Using a patient's own gene mutations as a vaccine may prove to be very useful in the future, although it remains difficult to translate into a widely available therapeutic because of the lack of consistent expression of the key mutations across the patient population.

9. DENDRITIC CELL VACCINES

Dendritic cell vaccines for colon cancer have generally been loaded with MAGE (a cancer antigen) or CEA. In one study, MAGE was expressed by one-third of colon cancers (48). In a larger study, MAGE expression was exclusive to the tumor tissue with at least one of the 10 MAGE antigens tested being present on 70 of the 80 samples and MAGE-3 expression was associated with increased metastatic potential (49). Clinical studies are ongoing using MAGE as a vaccine target in GI cancers. In one, Sadanaga et al. demonstrated the feasibility and safety of treating GI cancer patients with MAGE-3-pulsed autologous dendritic cells. Four of eight patients developed CTL responses and tumor markers decreased in seven patients (50). Minor regressions were seen in three patients, making this an extremely promising approach for the future. Fong and colleagues (51) administered Flt-3-ligand-mobilized dendritic cells loaded with the modified CEA peptide CEA610D to patients with CEA-expressing malignancies (predominantly colon cancer). The immunizations resulted in 2 of 12 patients with tumor regression, 1 patient with a mixed response, and 2 with stable disease. CEA-specific T cells identified by tetramer analysis were expanded in several patients and correlated with clinical outcome. Finally, Morse and colleagues have demonstrated the feasibility of administering CEA peptide and CEA-mRNA-loaded dendritic cells to patients with CEA-expressing malignancies (predominantly colon cancer). Current studies are utilizing fowlpox-CEA-TRICOM-infected dendritic cells as vaccines in this patient group.

10. SUMMARY

The work that is summarized above represents several decades of research and improved understanding of the immune system and its role in cancer therapy. We are now seeing more and more clinical responses in GI cancer patients, increased recognition of the importance of chemotherapy on the immune response, and consistent data from a number of different approaches suggesting the immune response correlates with clinical outcomes. GI cancers are clearly an important area in which to test vaccine agents. There is a significant positive research history, ample patients, ample ideas to test, and ideal clinical scenarios in which novel agents should be tested. I would predict that the next decade will mark the clinical establishment of vaccine approaches in GI cancers.

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Vaccines for Lung Cancer

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1. INTRODUCTION

The disappointing long-term survival for non-small-cell (NSCLC) and small-cell (SCLC) lung cancers has highlighted the need for additional strategies. The modest activity of therapies targetting epidermal growth factor receptor (EGFR) suggests that other targeted therapies such as immunotherapy are worthy of consideration. Several recent reviews have addressed this topic (1,2). Although renal cell carcinoma and melanoma are the classic targets for immunotherapy, attempts to activate the immune system against lung cancer have actually been ongoing for 30 yr or more. The initial approaches used bacterial cell wall products to cause a profound, but nonspecific, inflammatory response. Occasional studies demonstrated clinical benefit, and some of these agents are still being tested. Modern theories regarding the role of antigen-specific immune response, though, have given greater impetus to develop vaccines that activate immune responses against specific tumor antigens. This chapter will focus on the role of vaccines to activate immune responses against lung cancers.

2. IMMUNITY IN LUNG CANCER PATIENTS

2.1. Impairment of Immunity

As is the case for other solid organ malignancies, subtle abnormalities in components of the immune system may be demonstrated in some patients with lung cancer, even those

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with a very good performance status or low tumor burden. Twenty percent of newly diagnosed patients have been reported to be anergic and this correlated with lower survival (3). Defects in interleukin (IL)-2 secretion, abnormalities in the CD4/CD8 ratio, or diminished CD4+ helper cells in regional lymph nodes have all been reported (4–8). Although these abnormalities are not severe enough to lead to opportunistic infections as reported in patients with immunodeficiency syndromes, they may limit the ability of the immune system to control tumors.

2.2. Presence of Antitumor Immunity in Lung Cancer Patients

Despite immune defects, antitumor immune responses can be detected in some lung cancer patients. For example, more than half of SCLC and NSCLC patients have been described to have circulating antibodies reactive with autologous tumor proteins (9). Presence of these antibodies predicts better response to therapy and survival in some studies (9–11), whereas others, such as those specific for p53, are associated with a survival effect in some but not other studies (12–20). Detecting *in vivo* antigen-specific T-cell responses in lung cancer patients has been more challenging. Lymphocytes from regional lymph nodes draining lung cancers proliferate in response to autologous tumor to a greater extent than do peripheral blood lymphocytes (8,21), suggesting enrichment for tumor-specific effectors in regional lymph nodes. Tumor-infiltrating lymphocytes from lung cancers have been discovered to have a restricted T-cell receptor V-beta usage (22), a marker for the oligoclonality expected with an antigen-specific immune response. Cytotoxic T lymphocytes (CTLs) that can recognize autologous tumor have also been identified in lung cancer patients (23). T cells specific for the tumor antigen HER2/neu have been identified in patients with NSCLC (24). Finally, it has been possible to activate tumor antigen-specific T cells from lymphocytes obtained from peripheral blood, tumor specimens, or regional lymph node lymphocytes of healthy volunteers and lung cancer patients (25–27).

3. TUMOR ANTIGENS EXPRESSED BY LUNG CANCER

Numerous tumor antigens have been described for lung cancers. Among the most frequently expressed are the MAGE antigens with MAGE-1 reported in 11–36%, MAGE-2 in 30%, MAGE-3 in 38%–60%, and MAGE-4 in 13% of NSCLC (28–31). Using tissue microarray technology, expression of tumor-associated antigens (TAAs) of the MAGE family have been reported in 50% of squamous cell carcinomas of the lung and in 38% of large-cell carcinomas of the lung (32). In another study, MAGE-A10 was expressed only by SCLC, and MAGE-A1, 3, 6, 12, and 4b were expressed by both SCLC and NSCLC (33). CTLs derived from regional lymph nodes of lung cancer patients can recognize MAGE peptide epitopes (34). Other described antigens include NY-ESO-1 (35,36), Wilms' tumor-1 (WT1) (37,38), translation initiation factor eIF-4G (in squamous cell carcinomas) (39), carcinoembryonic antigen (CEA) (38), and Mac-2 binding protein (40). Overexpression of HER2/neu is controversial. Although reported in 4–60% of NSCLC (primarily adenocarcinomas) and 0% of SCLC, overexpression by fluorescence *in situ* hybridization (FISH) is rare (41–43). EGFR is overexpressed in virtually all squamous carcinomas and is also found in >65% of large-cell and adenocarcinomas, but not by SCLC (44,45). Finally, as is the case for other epithelial malignancies, the mucins, MUC1 and MUC4, have been identified in NSCLC (46,47).

4. NONSPECIFIC, ACTIVE IMMUNOTHERAPY

Although the purpose of this chapter is to describe the results for vaccines in the treatment of lung cancer, it is important to consider that nonspecific inflammatory mediators have demonstrated activity in lung cancer and although experimentally, they are giving way to the more specific tumor vaccines, they may be combined with vaccines in the future. Initial studies utilizing the bacille Calmette-Guérin (BCG) strain of *Mycobacterium tuberculosis*, bovis BCG, administered by the intrapleural, intratumoral, intra-dermal, or aerosolized routes demonstrated positive results, although two randomized trials failed to show any effect (48,49). Other attempts at nonspecific immunotherapy in lung cancer have included Nocardia rubra cell wall skeleton, *Corynebacterium parvum*, “transfer factor” (TF), Krestin (PSK), the streptococcal preparation OK-432 (50), and more recently, SRL172, a suspension of heat killed *Mycobacterium vaccae*, which is currently in a pilot study for SCLC (51). Patients with previously untreated NSCLC and mesothelioma were randomized to receive either chemotherapy alone, or chemotherapy given with monthly intradermal injections of SRL172. There was a trend toward improved response rate (54% vs 33%), median survival (9.7 vs 7.5 mo), and 1-yr survival (42% vs 18%) in the group of patients randomized to receive chemotherapy combined with SRL172 (52).

5. ACTIVE SPECIFIC IMMUNOTHERAPY

5.1. Tumor Cell Vaccines

As is the case with other solid tumors, lung cancer by themselves may be poorly immunogenic, but can become potent vaccines if modified to increase their immunogenicity. For example, modification of lung cancers with the costimulatory molecule CD80 results in a greater ability to activate CTL in vitro (53). In a phase I/II trial for patients with stage IV or relapsed NSCLC, an allogeneic, irradiated lung adenocarcinoma cell line transfected with CD80 and human leukocyte antigen (HLA)-A1 or -A2 was administered subcutaneously to 12 patients (54). There was one partial response and three with stable disease lasting 7–12+ mo. Three of these patients also showed significant increases (44- to 267-fold) in tumor-specific CD8 CTLs, measured by interferon (IFN)-gamma ELISPOT assay. Nemunaitis and colleagues (55) performed a phase I/II study of autologous lung cancers modified with an adenoviral vector encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) (GVAX). A challenge for these studies is obtaining an adequate number of cells and successfully modifying a majority of the tumor cells. In this study, 78% of the tumors were successfully modified and a median dose of 1.3×10^7 cells were produced. At the time of the report, 12 patients had been treated and 6 completed the immunizations. Of three patients with advanced measurable disease who completed the therapy, there was one complete response, one minor response, and one stable disease. A possible solution to the complexities of reliably modifying autologous tumor is to mix autologous tumor cells with bystander cells (K562) that are gene modified to secrete GM-CSF (56). The bystander cells produce much higher amounts of GM-CSF with less lot-to-lot variability. A solution to the difficulty in obtaining adequate numbers of autologous tumor is to use allogeneic cell lines transfected with immunostimulatory genes.

5.2. Immunization with Tumor Antigens

The availability of identified tumor antigens for lung cancers has made it possible to immunize patients directly against these antigens. The earliest studies used TAAs derived from cell membranes of various tumors. These had been found to induce antibody responses. In a phase III study of resected (stage I/II) squamous cell carcinoma, patients were randomized to TAAs emulsified with complete Freund's adjuvant (CFA), CFA alone, or no immunization. The median and 5-yr survival of the TAA group (106 mo and 75%) exceeded the CFA alone (71 mo and 53%), and no immunotherapy (34% and 38 mo) groups (57).

Because these were relatively crude preparations, regulatory considerations have driven the switch toward the use of more purified epitopes delivered either in adjuvant or presented by dendritic cells (DCs). In one clinical trial, HLA-A2+ patients with NSCLC and tumor expression of NY-ESO-1 were given eight weekly intradermal injections of NY-ESO-1 peptides 157–165 and 157–167 with GM-CSF as adjuvant (36). Preliminary results in four patients enrolled to date showed significant enhancement of NY-ESO-1 peptide 157–165 specific CD8+ T-cell reactivity. One patient had stable disease at the end of the study, whereas three patients did not have measurable disease. MUC-1 peptides such as the lipid-encapsulated 25-mer BLP-25 are under evaluation in lung cancer (58). Fixed-anchor and heteroclitic analog epitopes from p53, CEA, HER2/neu, and MAGE2/3 are also being developed for use in vaccines targeting lung cancer by Epimmune (San Diego, CA).

Several clinical trials utilizing DCs are under way in lung cancer patients. In one patient with a lung adenocarcinoma, we activated CEA-specific CTL immune responses by administration of an autologous DC vaccine loaded with total mRNA from their CEA-expressing tumor (59). In another clinical study of immunization with CEA peptide-loaded DCs, 2 of 10 HLA-A24 patients with CEA-expressing advanced digestive tract and lung cancers developed delayed-type hypersensitivity (DTH) responses to CEA (60). In two of these patients, disease remained stable for 6 and 9 mo. In a study of 12 patients with CEA-expressing colon and lung cancer, vaccination with Flt-3-ligand mobilized DCs loaded with a modified CEA resulted in 2 partial responses, 1 mixed response, and 2 with stable disease (61). Clinical activity correlated with development of T cells specific for the CEA peptide as identified by major histocompatibility complex (MHC)-peptide tetramers. In a phase I study, dexosomes, bilamellar particles released by DCs, loaded with MAGE peptides were administered to patients with stage III and IV NSCLC following their standard chemotherapy (62). The immunizations were well tolerated. Increased DTH reactivity to some MAGE antigens was observed. Three of nine patients remained stable following the immunizations. A phase II study of this approach in lung cancer patients has been proposed.

Anti-idiotypic vaccines, such as BEC2, which mimics the ganglioside GD3 expressed on the surface of most SCLC tumors, have been developed to target SCLC. Grant and colleagues (63) administered BEC2 plus BCG to 15 patients who had completed standard therapy for SCLC. All patients developed anti-BEC2 antibodies and five developed anti-GD3 antibodies, including those with the longest relapse-free survival. The median relapse-free survival for patients with extensive stage disease was 11 mo and had not been reached for patients with limited-stage disease (>47 mo). A phase III trial is being conducted to evaluate BEC2 plus BCG as adjuvant therapy after chemotherapy and irradiation for SCLC. A bivalent ganglioside vaccine, BMS248967, is in phase II studies (64).

6. SUMMARY AND CHALLENGES TO VACCINES FOR LUNG CANCER

Lung cancers, like other malignancies, can escape immune recognition by downregulation of HLA class I molecules. Administration of IFN-gamma has been shown to upregulate HLA class I on NSCLC and thus may be a useful adjunct (37). Fas-ligand (Fas-L), frequently detected in lung carcinoma cell lines and resected tumors, can cause apoptosis of T cells. In fact, lung carcinoma cells were capable of killing the Fas-sensitive human T-cell line Jurkat in coculture experiments (65). Inhibiting Fas-L expression by tumors might therefore be a useful adjunct to immunotherapies. The tumor antigen RCAS1, positive in 47% of NSCLC specimens (and up to 80% of adenocarcinomas), induces apoptosis in immune cells bearing the RCAS1 receptor (66). Interference with its activity might improve the activity of lung cancer immunotherapy. Lung cancers may negatively regulate DC differentiation and this observation supports the use of ex vivo-generated DCs in vaccine strategies (67). Lung cancer cell lines express higher levels of proteins that can interfere with complement activity such as membrane cofactor protein (MCP; CD46), decay-accelerating factor (DAF; CD55), and CD59. Resistance to complement-mediated lysis of the lung cancer cell lines was much higher than that of normal cells (68). Inhibition of complement activity may allow lung cancers to avoid antibody-mediated cytotoxicity.

Inhibition of T-cell activity is also common in the setting of lung cancer. Woo observed high levels of CD4(+)CD25(+) regulatory T cells in lung cancers (69). These T cells mediate potent inhibition of autologous T-cell proliferation and suggest a mechanism whereby lung tumors may inhibit the host immune response (69). Monoclonal antibodies that can deplete CD25+ T cells may therefore be of use in inhibiting the counterregulatory response.

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Vaccines for Other Tumors

Michael A. Morse, MD

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1. CNS GLIOMAS

Although the brain is often considered an immunologically privileged site, animal models have demonstrated immunity against brain tumors following peripheral immunizations (1), direct intracerebral injections (2), or both (3). Potential targets of immunity against gliomas include the antigens ADP-ribosylation factor 4-like (ARF4L) (4), SART1 (5) and SART3 (6), UDP-Gal: β GlcNAc β 1, 3-galactosyltransferase, polypeptide 3 (7), and the VIII mutation of human epidermal growth factor receptor. Although the autoimmune condition, experimental allergic encephalomyelitis, is a theoretical risk for immunizations with central nervous system (CNS) antigens, it is believed that the use of tumor-specific antigens will limit this risk.

Human clinical trials with vaccines for gliomas are thus far limited. In a phase I trial, nine patients received intradermal immunizations with dendritic cells (DCs) loaded with tumor-derived peptides following surgical resection and radiotherapy of their gliomas (8). Cytotoxic activity was demonstrated following the immunizations in four of seven tested patients including intratumoral cytotoxic and memory T-cell infiltration in two of four patients. The Duke University Medical Center Neuro-oncology group is currently performing a study using DCs loaded with PEP-3, a peptide fragment spanning the tumor-specific mutation of the EGFRvIII. Immunologic monitoring data from this trial have confirmed that PEP-3-KLH-pulsed DCs can elicit a potent immune response in patients with gliomas without inducing autoimmunity (Sampson, personal communication).

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Because radiotherapy is an important component of the treatment of CNS tumors, it will be of interest to determine whether human studies will demonstrate similar promising results as demonstrated in animal models combining cranial irradiation and anticancer immunization (9).

2. MEDULLARY THYROID CANCER

Expression of carcinoembryonic antigen (CEA) by medullary thyroid carcinoma (MTC), a neoplasm that arises from the parafollicular cells of the thyroid, has been used as a target of radioimmunotherapy. More recently, cancer-testis antigens, in particular NY-ESO-1, have been detected in medullary thyroid cancer (10). Thus, vaccines targeting these antigens may be useful for treatment of this malignancy. Also, because medullary thyroid cancer frequently arises as a familial syndrome, one interesting approach would be to apply immunizations as a way to prevent development of the disease as has been demonstrated in an animal model of familial human MTC (11).

3. PARATHYROID CARCINOMA

Parathyroid carcinomas continue to produce parathyroid hormone (PTH), which can serve as a tumor antigen. In an innovative approach, an immune response was activated against the PTH secreted by a patient with metastatic parathyroid carcinoma (12). This patient was immunized subcutaneously and intralymphatically with mature tumor lysate and parathyroid hormone-pulsed DCs. Keyhole limpet hemocyanin (KLH) was later added for CD4+ help. T-cell proliferative responses and delayed-type hypersensitivity (DTH) reactivity specific for the tumor lysate were generated.

4. NEUROBLASTOMA

Neuroblastomas express several identified tumor antigens including the ganglioside GD2, MAGE 1-4, GAGE, and the MYCN oncogene. Indeed, cytotoxic T lymphocytes (CTLs) activated by the MYCN-derived human leukocyte antigen (HLA)-A1-restricted S9K peptide (STMPGMICK), specifically lysed HLA-matched, MYCN-amplified neuroblastoma tumor cells (13). Immunologic responses against neuroblastoma have been well documented. In a clinical trial of 21 patients with relapsed or refractory neuroblastoma (14), immunizations with lymphotoxin- and interleukin (IL)-2-secreting allogeneic neuroblastoma cells induced a complete remission in 2 patients and partial response in 1 patient. Interestingly, the immune response detected was actually a natural killer (NK) and a T helper-2 (Th2) response with an associated increase in tumor-specific immunoglobulin G (IgG), suggesting that the clinical responses may have been due to antibody rather than CTL responses. In a phase I trial of tumor lysate-pulsed DCs in 15 children with relapsed or refractory solid tumors including neuroblastoma and sarcomas (15), doses of 1×10^6 to 1×10^7 tumor lysate and KLH-loaded, immature DCs were administered intradermally every 2 wk for a total of three vaccinations. The clinical results included one major clinical response, two with stable disease, and three with no evidence of disease who remained disease free for 16–30 mo. Tumor-specific T cells secreting interferon- γ were detected from unstimulated peripheral blood mononuclear cells in three of six analyzed patients. Finally, it has been observed that the activity of the anti-GD2 monoclonal antibody 3F8, which demonstrated benefit in patients with stage

IV neuroblastoma, may have been because of the activation of an anti-anti-idiotype network (16).

5. HEAD AND NECK CANCERS

Head and neck cancers express a number of antigens including CEA (17), SSX, RAGE, and GAGE. Unfortunately, patients with squamous cell carcinoma are frequently nutritionally depleted and thus may not be ideal candidates for immunotherapy. Thus far, immunotherapy for head and neck cancers has been fairly limited and has consisted primarily of tumor-activated T-cell infusions. In a phase I study, six patients with advanced head and neck cancers received infusions of vaccine-primed lymph node cells activated by immunization with irradiated autologous tumor cells admixed with bacille Calmette -Guérin (BCG) (18). Although tumor-specific CD4+ and CD8+ responses were activated, there were no tumor responses after cell transfer. In another phase I study, 17 patients with recurrent or metastatic squamous cell carcinoma of the upper aerodigestive tract received lymph node lymphocytes activated by immunization with irradiated autologous tumor cells admixed with granulocyte-macrophage colony-stimulating factor (19). Three patients had stable disease and one patient remained free of disease 4 yr after resection of a vertebral body metastasis.

Nasopharyngeal carcinoma, which is associated with Epstein-Barr virus (EBV) infection, in contrast, may be an excellent target for immune therapy. In a study of 16 patients with advanced nasopharyngeal carcinoma, intranodal injections of DCs loaded with EBV latent membrane protein (LMP)-2 peptides activated epitope-specific CD8+ T-cell responses in 9 patients (20 Lin). Epitope-specific cytotoxicity could be detected in the peripheral blood 3 mo after immunization. Two patients experienced a reduction in tumor burden.

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IV

CLINICAL TRIALS AND IMMUNE ANALYSES

34

Clinical Trial Designs for Therapeutic Vaccine Studies

Richard Simon, DSC

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1. INTRODUCTION

Cancer vaccines are different in many ways from cytotoxic drugs. Consequently some of the paradigms for the early clinical development of cytotoxics are not applicable to the development of therapeutic vaccines. In contrast, many of the principles of phase III clinical trials are applicable to cancer vaccines. In this chapter we will describe design strategies for the efficient early clinical development of cancer vaccines.

2. PHASE I CLINICAL TRIALS

The major objective of phase I trials of cytotoxics is determining a dose of the drug or regimen that is safe for use in subsequent clinical studies. For cytotoxics it is assumed that higher doses have greater antitumor effects and so phase I trials are usually designed to estimate the maximal tolerated dose.

Tumor vaccines are often based on DNA constructs, viral vectors, and cytokines that have been determined as safe from previous clinical trials. Peptide vaccines generally seem inherently safe so long as the cytokine adjuvants are used in combinations and doses previously demonstrated to be safe. For example, peptide vaccines based on nonmutated melanoma antigens such as MART-1/Melan A and gp100 were initially evaluated in a phase I trial at the National Cancer Institute at doses ranging from 0.1 to 10 mg. However, no toxicity was encountered even at the highest dose (1,2).

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Table 1
Finding the Minimum Active Dose

<i>Probability of Immunologic Response</i>	<i>Number of Patients Treated at Dose</i>	<i>Probability of No Immunologic Responses</i>
0.20	11	0.09
0.25	9	0.08
0.30	7	0.08
0.40	5	0.08
0.50	4	0.06

On the other hand, initial clinical experience with a novel virus or plasmid as a recombinant vaccine vector should be conducted in a phase I setting. However, if such vectors are proven to be nontoxic even at substantial doses, then subsequent trials using the same vectors but with different recombinant inserts may not require dose-escalation studies.

For cancer vaccines, it is not always the case that more is better. For example, in studies of peptide vaccines based on nonmutated melanoma antigens, *in vitro* analysis did not reveal any correlation between peptide dose and the generation of specific T-cell reactivity from the peripheral blood lymphocytes of vaccinated patients (1,2). Thus, for subsequent trials using similar peptides, an intermediate fixed dose of 1 mg was chosen for vaccination, bypassing repetitive phase I studies.

Feasibility issues limit the maximum doses of certain recombinant proteins, viruses, or whole-tumor-cell vaccines that can be produced for administration to patients. In many cases, the dose selected will be based on preclinical findings or on practical considerations.

Dose ranging to find the minimal active dose may be feasible but the three to six patients per dose level used in conventional toxicity trials may not be adequate. Those small sample sizes are only sufficient to exclude high toxicity rates. Suppose that an assay is used in a binary manner to define immunogenic response. Table 1 shows the probability of no immunogenic responses in n patients as a function of the true immunogenic response probability. If one wants a dose at which the immunogenic response probability is at least 30% say, then if you observe no immunogenic responses in seven patients it would be appropriate to escalate to the next dose level. Simon's optimal two-stage phase II designs described below, or other phase II designs used more generally to distinguish a response probability of less than p_0 (e.g., 0.05) from one greater than p_1 (e.g., 0.30). But unless p_1 is much greater than p_0 , the required number of patients will be much larger than for cytotoxic phase I trials (*see* Table 2).

Trying to determine whether there is a dose-response relationship involves comparing immunological responses for different dose levels. Such trials, if designed properly, require larger sample sizes. For example, suppose one wishes to plan a study of two dose levels and test whether there is a relationship between dose and immunologic response. If the immunologic response probabilities at the two dose levels are 50% and 90%, then 20 patients treated at each dose level are required for a one-sided statistical significance level of 0.10 and a statistical power of 0.90 (3). Larger sample sizes are required to detect smaller differences. Using more than two dose levels allows one to treat somewhat fewer patients at each dose level, but the total number of patients required to detect a dose-

Table 2
Optimal Two-Stage Designs

<i>Target Response Rate (p_1)</i>	<i>First Stage Sample Size (N_1)</i>	<i>Maximum Sample Size (N)</i>	<i>Number of Responses Required for Activity (A)</i>	<i>Probability of Early Termination</i>
20%	12	37	4	.54
25%	9	24	3	.63
30%	7	21	3	.70
35%	6	12	2	.74

Adapted from ref. 8.

response relationship will actually be much larger than if only two dose levels are tested. This is because the two most extreme dose groups are the most informative for detecting a dose-response relationship.

Trying to characterize the shape of the dose–activity relationship or finding an optimum biologic dose is an even more ambitious objective that is rarely practical in a phase I tumor vaccine study.

Phase I trials of cytotoxics are generally conducted in end-stage patients for whom all established therapy has been exhausted. End-stage patients without intact immune systems may have very little likelihood of benefit or toxicity from a tumor vaccine, however. In some cases the potential toxicity of the regimen may be based on immune stimulation and will not be seen in anergic patients. Hence, such patients contribute little information about potential toxicities for individuals with intact immune systems.

3. PHASE II CLINICAL TRIALS

The objectives of the phase II vaccine trial are similar to those of the phase II cytotoxic trial. The primary objective is to determine whether the regimen has biologic activity that is likely to translate into patient benefit. The second objective is to optimize the regimen so that the biologic activity is likely to translate into patient benefit in phase III trials.

With cytotoxics, the generally accepted endpoint for phase II trials is objective tumor response, that is, tumor shrinkage by at least 50%. Tumor shrinkage is not a direct measure of patient benefit, although it sometimes is predictive of benefit. The most commonly accepted direct measures of patient benefit are survival, disease-free survival, and symptomatic relief. Therapeutic effect on these end points cannot be reliably established outside of a phase III trial with an appropriate control group not receiving the experimental therapy. Investigators sometimes like to infer that a regimen prolongs survival because the responders live longer than the nonresponders, but this analysis has long been known to be invalid (4,5).

Tumor shrinkage is generally used as the end point for phase II trials of cytotoxics for two reasons. First, response represents biological activity that can be attributed to the therapy (i.e., tumors rarely shrink spontaneously by 50%). Second, if the degree, duration, and abundance of responses are sufficient, then it is plausible to hope that tumor response may translate into patient benefit. There are many cytotoxic regimens that were active in

phase II trials but that subsequently had no identifiable effect on survival in phase III trials. Torri et al. (6) performed a meta-analysis of randomized trials to quantify the relationship between improvement in response rate and improvement in survival outcome for advanced ovarian cancer studies. They found that a very substantial improvement in response rate was necessary to have any identifiable effect on survival.

For phase II tumor vaccine studies, the investigator has the choice of using clinical end points or immunological end points. Clinical end points include tumor shrinkage, reduction in tumor marker levels, or delay in time to tumor progression. Tumor shrinkage is always a welcome occurrence in a phase II tumor vaccine trial. The question is whether a regimen should be precluded from a phase III evaluation of early-stage patients if it does not shrink metastases in more advanced cases. Opinion is divided on this important point.

3.1. Tumor Shrinkage End Point

If tumor shrinkage is the end point, then phase II designs used for cytotoxics can be employed (7–9). Simon’s “optimal two-stage” designs are widely used for phase II cytotoxic trials to test whether a regimen has a response rate above a background level p_0 (8). Frequently, $p_0 = 0.05$ is used. With clinical response, this assumes that no more than 5% of the patients will have apparent responses caused by variability in response assessment or spontaneous remissions. The two-stage design incorporates an early termination point, which allows the investigator to discontinue patient accrual if a desired end point has not been achieved in the first stage of the trial.

At the conclusion of the clinical trial, the regimen will be declared active or inactive. Table 2 shows several designs with 10% false-positive rate, 10% false-negative rate, and $p_0 = 0.05$. The false-positive rate (α) is the probability of declaring the regimen active when the true response probability is p_0 . The false-negative rate (β) is the probability of declaring the regimen inactive when its true response probability is the target response rate p_1 , the level of activity that we wish to be able to detect. In the first stage, N_1 evaluable patients are entered and treated. If no responses are observed, then the trial is terminated and the regimen is declared inactive. Otherwise accrual continues to a total of N evaluable patients. At that point accrual is complete. If the total number of responses is at least A , then the regimen is declared active. The last column of the table indicates the probability of early termination after the first stage when the true response probability is p_0 . For example, if $p_0 = 5\%$ and the target response rate is 25%, then nine patients are treated in the first stage of the trial. If no responses are observed, the trial is terminated. Otherwise, accrual is continued to a total of 24 patients. If at least three responses are seen in the 24 patients, the regimen is declared active. The probability of declaring a regimen active when its true response rate is 5% or less is 10%. The probability of missing the activity of a regimen with a true response rate of 25% is 10%. With a regimen having a response rate of 5%, the probability of stopping after only nine patients is 63%.

This design with $p_1 = 25\%$ and $p_0 = 5\%$ seems reasonable for many initial vaccine trials but designs based on other parameter values are easily generated using computer program OTSD (optimum two-stage design) available at <http://lib.stat.cmu.edu/designs>. The required number of patients depends strongly on the difference $p_1 - p_0$. These designs are based on a binary measure of response. We will deal with time to progression end points in a later section.

3.2. Tumor Marker End Points

Traditional phase II trials in patients with clinically measurable tumors are often not considered appropriate as initial trials of whether tumor vaccines have biological effect. Vaccine trials are best conducted in patients with intact immune systems and for some diseases this precludes inclusion of patients with gross tumor.

The strategy of vaccination in a minimal disease state and using a sensitive tumor marker or molecular probe to measure reduction or disappearance of subclinical tumor mass can be an effective alternative for development of tumor vaccines. This approach was used in obtaining promising initial results for an idiotypic lymphoma vaccine that is now in randomized phase III testing using conventional clinical end points (10). Although the relevance of a molecular marker of subclinical disease to long-term prognosis may be in question, such a marker can provide a measure of antitumor effect that can be measured in patients with minimal residual disease and is thus useful for early vaccine trials.

3.3. Time to Tumor Progression End Point

Evaluating whether a treatment delays recurrence or progression is particularly important for tumor vaccines. Patients without clinical evidence of disease may have more intact immune systems and be more appropriate candidates for tumor vaccines than patients with more advanced measurable metastatic disease. This will depend, however, on the type of therapy used to put them in the clinically disease-free status.

Evaluating the effect on a regimen on time to progression of subclinical disease is particularly problematic in a single-arm phase II trial. It is easy to devise a definition of disease stabilization, i.e., lack of recurrence or progression for a specified period of time, but the validity of the definition depends on the existence of data that establish that such stabilization does not occur in the absence of treatment. This is difficult to establish reliably because of the usual difficulties of identifying comparable nonrandomized controls and because of special difficulties involved with measuring time to disease progression in a consistent manner for different cohorts of patients. Consequently, we believe that the use of disease stabilization or time to progression as an end point in single-arm trials should only be considered when data from a specific set of contemporaneous controls from the same institution are available. In such a case, rather than attempting to define disease stabilization in a valid manner as a dichotomous end point (e.g., present or absent based on some threshold), it is preferable to compare the time to progression for the patients in the phase II trial to the distribution of time to progression of a specific set of control patients not receiving the vaccine regimen. Dixon and Simon (11) provide formulas for computing the number of patients required in the single-arm trial.

Generally, single-arm trials of disease stabilization, time to tumor progression, or time to tumor recurrence are not sufficiently reliable. The randomized “phase 2.5” design described below is a more satisfactory approach.

Phase III trials are generally randomized comparisons of a new regimen compared to a standard treatment using an end point of established medical importance to the patient such as survival or quality of life. Phase III trials are usually planned using a 5% type I error parameter (α) because the results of phase III trials are viewed as definitive and are used as a basis for marketing approval and practice guidelines. We propose that in the development of cancer vaccines, there is a role for what might be called a “phase 2.5”

trial. Such a clinical trial would also be randomized, but may use an end point measuring biological antitumor activity even though the end point might not be established as a valid surrogate for survival or quality of life. The phase 2.5 trial might also be based on an elevated statistical significance level since the objective of the trial would not be for regulatory approval or for establishing general practice guidelines.

To detect a large effect of a treatment in delaying tumor progression in a rapidly progressive disease such as pancreatic cancer or melanoma with visceral metastases does not require many patients in a randomized trial. With exponentially distributed times to progression, a 40% reduction in the hazard of progression corresponds to a 67% increase in median time to progression. In order to have 80% power ($\beta = 0.20$) for detecting this size of effect using a traditional $\alpha = 0.05$, only about 117 patients are required (assuming accrual rate of about 3 patients per month, median time to progression of 12 mo for control group and follow-up time of 24 mo after end of accrual) (12). This can be reduced to 87 patients if $\alpha = 0.10$. Hence, 44 patients randomized to vaccine and the same number randomized to control, one can conduct a randomized phase 2.5 trial for evaluating whether the vaccine reduces the hazard of progression by 40%. This design would be a phase 2.5 design because of the unconventional use of a one-sided $\alpha = 0.10$ significance level and because time to progression might not be established as representing clear patient benefit. The phase 2.5 design is similar to the phase III design in the respect that it contains a control group for evaluating the experimental regimen and the intent is comparative.

The basis of the efficiency is that the disease is rapidly progressive and a large treatment effect is targeted. If the median time to progression for the control group were 6 mo instead of 12, an even smaller sample size would be required. When the disease is not rapidly progressive the efficiency illustrated here decreases. Statistical power for detecting a specified reduction of the hazard of an event is determined by the number of events, not the number of patients. With a slowly progressive disease, it may take many patients to be entered in order to observe a specified number of events unless the follow-up time following the close of accrual is very long. Also, for a rapidly progressive disease, a large reduction in hazard, e.g., 40%, is associated with a moderate absolute increase in median time to event; e.g., 6 mo increased to 10 mo, 12 mo increased to 20 mo. Consequently, with a rapidly progressive disease there is greater justification for targeting a relatively large treatment effect.

3.4. Immunological End Points

For some types of cancers, patients with measurable tumors and intact immune systems are available but existing vaccines do not produce clinical tumor responses. In such circumstances, immunological end points may guide attempts to optimize the vaccine and its delivery before randomized phase 2.5 trials or randomized phase III trials are conducted. A phase III evaluation of a regimen may not be warranted, however, until the regimen demonstrates activity based on a clinical end point.

When an immunological end point is used, the protocol should provide specific information about the variability of the measurement of that end point. Three sources of variability should be distinguished: variability among assay results on the same specimen (e.g., lymphocytes, serum, or tumor tissue), variability among specimens from the same patient drawn at different times, and variability among patients. Documenting this

variability is essential for interpretation of results of the trial. For example, such data permit one to define a threshold for change in the immunological end point that can be regarded as statistically significant. If these data are not available at the outset, it may be possible to develop them during the course of the clinical trial (e.g., from multiple baseline blood drawings for the patients to be vaccinated or from drawings for control patients). It is important to prepare plans for analysis of immunological end points in advance and include these plans in the protocol in order to ensure that needed data are available and to reduce subjectivity in the analysis.

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Analysis of Cellular Immune Responses in Cancer Vaccine Trials

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1. INTRODUCTION

Active immunotherapy seeks to stimulate therapeutic immune responses through the use of a wide variety of immunogens. Increasingly, scientific progress in the field has come to rely on the use of assays for detecting and quantifying antigen-specific immune activation and expansion, founded on the belief that these assays may be correlates of in vivo antitumor activity. This is based upon the assumption that induction of an effective antitumor immune response should lead to measurable immune responses. Assays that accurately portray the characteristics of antitumor immune responses would increase our understanding of the effector mechanisms relevant to antitumor activity and help guide the further development of the current vaccines and the next generation of immunotherapeutic strategies. Additionally, accurate immunologic assays could provide intermediate end points for the early evaluation of vaccines and vaccine adjuvants and might provide insight into clinical observations including toxicities and antitumor responses. This perceived link between immunologic assay measurements and antitumor response has not been clearly demonstrated for most current assays of cellular immunologic responses.

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A wide variety of immune assays now exists. The choice of immune assay used depends on the expected mediator of the response. Conceivably, the most potent cancer vaccines will simultaneously activate multiple pathways and effectors that play complementary roles. Important effectors include CD8+ cytotoxic T cells (CTLs), which directly recognize tumor peptides presented by major histocompatibility complex (MHC) molecules on the surface of a tumor cell, triggering direct cytolysis, CD4+ helper T cells, particularly T helper type 1 (Th1) responses, that lead to CTL generation. Other effector cells such as macrophages and eosinophils, and humoral responses may also be important in antitumor immunity. This review will focus on T-cell-mediated immune responses, discussing the extensive variety of assays available to measure them.

A number of assays show promise as methods for quantifying and characterizing the T-cell response to immunizations and for serial monitoring of these responses. These tests can be grouped into three categories: in vivo functional measures, in vitro phenotypic assays, and in vitro functional assays. Important features of an assay for detecting T-cell responses include: (a) adequate sensitivity, specificity, reliability, and reproducibility, (b) measurement of the true state of in vivo T-cell activity without introducing significant distortions, (c) simple and rapid to perform, (d) requirement for only small quantities of specimens, and (e) close correlation with clinical data.

2. T-CELL SUBSETS

An emerging issue in the immune monitoring of cancer vaccine trials is the increasing understanding of the complexity of T-cell subsets and their different impacts upon immune responses. Recently, studies of T-cell clones, first in the mouse and subsequently recapitulated in the human, have led to the characterization of specialized subsets of Th cells and CTLs. Specific cytokine secretion profiles now permit the subdivision of Th cells into Th1 and Th2 subsets, with interferon- γ (IFN- γ) and interleukin (IL)-12 being the principal Th1 cytokines and IL-4 and IL-10 being the principal Th2 cytokines. Th1 cells are thus associated with induction of CTL responses, whereas Th2 cells are involved in promoting antibody responses. The cytokines produced by each cell type regulate the other cell type.

Indeed, the effect of IL-4 on Th1 cells and IFN- γ on Th2 cells is antagonistic, such that each subset essentially suppresses the proliferation of the other subset. As a consequence, Th2 cells could negatively impact CTL generation and the maintenance of CTL responses that are the objective of cancer vaccines, and evidence is mounting that they could also play a role in cancer growth, because some cancer patients have a predominant Th2 bias (1,2). These two Th cell subsets have led to similar definitions being drawn for CTLs, specifically TC1 and TC2, and dendritic cells (DCs), specifically DC1 and DC2. Again, type 1 cells are involved with promoting CTL responses and type 2 cells are involved in the generation of antibody responses. It should also be noted that the definition of cell types has broadened as further subsets of cells have been categorized. For example, there are now five and perhaps six Th2 subsets (3). Immunologic monitoring of cancer vaccine clinical trials until very recently has concentrated on CTL responses (mostly TC1 in nature). Now emphasis is also being placed on examining Th2 responses. This review will consequently focus on assays for measuring immune responses in both of these T-cell subsets.

3. IN VIVO MEASURES OF ANTIGEN-SPECIFIC IMMUNITY

3.1. *Delayed-Type Hypersensitivity*

In the delayed-type hypersensitivity test (DTH), an intradermal injection of antigen in the form of soluble protein alone or as antigen loaded onto antigen-presenting cells is administered and the diameter of any resulting erythema or induration after 48–72 h is measured. CD4+ Th cells that recognize the antigen presented on local antigen-presenting cells mediate the DTH response by releasing inflammatory, Th1 cytokines that increase vascular permeability and recruit monocytes and other inflammatory cells to the injection site. Occasionally, a similar response may be mediated by CD8 + T cells (4). The definition of what represents a positive DTH response has not been standardized nor has the dose for DTH testing, although protein antigens are generally administered as 10–50 µg in 0.1 mL. This low dose is considered small enough that it does not induce an immune response or cause excessive skin toxicity, but is of a sufficient magnitude to induce a detectable local response.

DTH remains one of the most frequent immune tests performed in immunotherapy studies (5–7), in part because it is straightforward to perform and may provide an *in vivo* surrogate of antitumor T-cell responses, but a number of questions remain regarding its value. The first is whether the DTH response is truly antigen-specific. Thurner (8) vaccinated patients with peptide-loaded DCs and detected induration and erythema at the injection site in 7 of 11 patients, but also found similar results for DCs not loaded with any antigen. In our own studies, some patients with no obvious induration or erythema had inflammatory infiltrates at the injection site in skin biopsies taken after DTH testing with DCs loaded with carcinoembryonic antigen (CEA) peptide (9). Other components of the immunizing agent may also contribute to the DTH response. For example, intradermal granulocyte-macrophage colony-stimulating factor (GM-CSF), a component of some vaccine strategies, by itself may induce a GM-CSF-specific DTH reaction (10). Some authors have grown the cells to be used for immunization in fetal bovine serum, which contains proteins that may serve as potential immunogens (11). The second issue is whether the dose of immunizing antigen correlates with immune response. For example, Schreiber (6) observed that the diameter of erythema and induration at an autologous, unmodified tumor injection site increased with each dose (except the last) and was greater with higher cell doses, suggesting the possibility of correlating dose and immune response. Third, although there is some evidence that suggests a concordance of DTH with other immune assays, it has not been clearly established. Disis and colleagues (12) observed that DTH induration of ≥10 mm (but not 5–9 mm) correlated with a positive HER-2/neu-specific T-cell proliferative response (stimulation index >2.0) in patients immunized with HER2/neu peptides. Fourth, a correlation of clinical outcome with DTH response has not been proven, since the number of patients studied in any one trial has been small. Nestle (13) immunized melanoma patients with DCs loaded with MAGE-1, MAGE-3, MART-1, gp-100, or tyrosinase peptides along with keyhole limpet hemocyanin (KLH) by direct intra-lymph node injection. Nine of the 12 immunized patients developed DTH responses to peptide-loaded DCs and 5 had diameters greater than 10 mm; 4 of these patients had clinically detectable tumor regression. Because it is relatively straightforward to perform and may possibly serve as an *in vivo* measure of the trafficking of lymphocytes to sites of tumor antigen, DTH testing will likely remain part of the immune

analysis of cancer vaccines. The ability to isolate, expand, and assess the phenotype or function of antigen-specific T cells from skin biopsies of DTH sites may serve as an additional strategy to evaluate antigen-specific immune responses (14).

4. IN VITRO MEASURES OF IMMUNE RESPONSE

4.1. Sources of Lymphocytes for In Vitro Immune Analysis

The most appropriate source of T lymphocytes for immune analysis is the subject of considerable debate. Clearly, peripheral blood is the most convenient source of T cells to sample, but it is also possible that peripheral blood T-cell activity may not represent the true effector population following immunization. It has furthermore been suggested that peripheral blood T-cell activity may not correlate with clinical response (15). Using human leukocyte antigen (HLA)/peptide tetramers, Lee and colleagues (15) enumerated melanoma antigen-specific T-cell precursor frequency directly in peripheral blood mono-nuclear cells (PBMCs) from melanoma patients vaccinated with gp100 peptide with or without IL-2. Although no antigen-specific T cells could be cultured ex vivo from PBMCs of IL-2-treated patients, these were the only individuals in whom tumor regressions occurred.

Whereas tumor tissue may contain lymphocytes specific for tumor antigens (16), the detection of a lymphocytic infiltrate in a tumor has not uniformly correlated with an improved prognosis in cancer patients and tumor-infiltrating lymphocytes (TILs) may have defects in the expression of T-cell receptor (TCR)-associated molecules (such as the CD3 zeta chain) (17). Regional lymph nodes draining from the site of immunization may contain the most recently stimulated T cells, but it has been shown that even healthy, non-tumor-bearing individuals may have lymph nodes harboring MART-1-specific T cells (18). T cells specific for the antigen of interest have been cloned from DTH sites; although this may serve as a surrogate for tumor infiltration, the conditions at a skin injection site not infiltrated with tumor are unlikely to be like the tumor tissue itself.

Despite the theoretical concerns with sampling only peripheral blood lymphocytes, because of the ease of sampling and the ability to perform assays on multiple time points, a significant amount of effort has focused on detecting circulating antigen-specific T-cell responses as discussed below. It is important to have prevaccination samples to establish the baseline immune response prior to immunization. In addition, the timing of the postvaccination samples can have a major impact on establishing the magnitude and durability of the measured response. Handling conditions of the specimens also influences results and must be considered. Cryopreservation of specimens for later analysis is convenient but may significantly decrease the detectability of an immune response, so analysis of fresh peripheral blood samples is also a consideration.

4.2. Analysis of T-Cell Receptor V Region Gene Usage and Complementarity Determining Region 3 Sequences

A very straightforward approach to measuring antigen specific T-cell responses would be to use easily identified phenotypic markers for the response. Specimens of lymphocytes obtained before and after an immunization strategy could be tested using standard flow cytometric methods. By exploiting the ability to phenotype T cells by the variable chain usage of their TCRs, it is possible to follow the expansions of specific TCR gene subfamilies of T cells following vaccination. Initial attempts to directly visualize antigen

specific T cells used combinations of antibodies that recognize different variable (V) region subfamily of the TCR alpha or beta chains. An increase in the number of cells expressing a particular V- α or V- β chain would denote development of oligoclonality, a possible sign of induction of a specific immune response (19). Unfortunately, this approach has limited value for a number of reasons. First, only a minority of T cells expressing a particular V- α or V- β combination will be specific for a particular antigen. Second, the response to most antigens is quite diverse and utilizes many different variable regions. Third, monospecific antibodies are not available for all V region gene subfamilies, especially the TCR α V region subfamilies, so this analysis is incomplete at best.

It has been suggested, for some antigens, that antigen-specific T cells have a restricted TCR repertoire (20) that can be detected by sequencing the third complementarity-determining region (CDR3) of the TCR. The CDR3 region encodes the highly polymorphic portion of the TCR responsible for recognizing peptide–MHC complexes. For the β chain, the CDR3 region encodes the Variable (V) region-Diversity (D) segment and Diversity segment-Joining (J) segment junctions, whereas for the alpha chain it encodes the V-J junction. Using V, D, or J-region subfamily specific polymerase chain reaction (PCR) primers, PCR may be performed to detect the development of restricted TCR gene usage (21,22). There is compelling evidence for restricted TCR V-region usage in the response to viral diseases (23,24). Some studies in melanoma patients (25,26) and renal cell carcinoma patients (27) have detected a restricted TCR gene usage. However, other studies in melanoma have found unrestricted TCR gene usage (28,29; Clay TM, unpublished observations). More studies are needed to determine the role of this new technology in monitoring immune responses in clinical trials. Advantages include the small amount of specimen required, the ability to perform the analysis from cells directly isolated from the blood to avoid introducing biases caused by ex vivo expansion, the reproducibility, and internal controls that permit analysis of samples collected at different times. Recently, a more automated and rapid fluorescence-based method for CDR3 length analysis of expressed TCR gene families that was able to distinguish between polyclonal, oligoclonal, and monoclonal CDR3 distributions has been developed (30).

4.3. Flow Cytometric Analysis of Antigen-Specific T Cells Using Peptide–MHC Tetramers

Recently, it has become possible to directly visualize antigen-specific T cells by using soluble multimeric MHC–peptide complexes. In 1997, Altman and Davis (31) demonstrated that fluorescently labeled, tetrameric peptide–MHC complexes could indeed bind stably, specifically, avidly to antigen-specific T cells. Another methodology for multimer generation involves using an antibody to link two peptide–MHC complexes, forming peptide–MHC dimers, often termed MHC–Ig complexes (32,33). In theory, only T cells with receptors specific for the peptide used in the complex will be recognized by these approaches. Using standard flow cytometric analysis, one can gate on the T cells and look for expression of the antigen-specific TCR. Analysis of peripheral blood T cells specific for potent immunogens such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV) demonstrated that between 0.2 and 6% of circulating CD8+ cells were specific for peptides representing these antigens. The quantitation of antigen-specific CD8+ T cells by flow cytometry using peptide–MHC tetramers has been shown to correlate well with traditional in vitro cytotoxicity assays (34). In addition, the intensity of staining of CD8+ T cells with peptide–MHC tetramers appears to correlate well with T-cell avidity for the

antigen (34). A number of studies have shown the utility of flow cytometric analysis using peptide–MHC tetramers to quantitate CD8+ T cells specific for tumor antigens or control antigens often used in immunotherapy protocols (15,35,36). Dunbar and colleagues (37) used peptide–MHC tetramers to isolate antigen-specific T cells from peripheral blood or lymph nodes by cell sorting. Following cloning of these selected T cells they were shown to respond to specific antigen by cytokine production. However, it is not known whether simple binding of T cells to peptide–MHC tetramer accurately predicts functional activity.

Although peptide–MHC tetramers are powerful tools, they have certain limitations. In particular, they can only be used to detect known immune responses, as the peptide of interest must be loaded into the peptide–MHC tetramer and thus must already be known and synthesized. Immune responses to unknown antigens thus cannot be detected. Additionally, only class I MHC tetramers have been routinely available for widespread use; construction of class II tetramers has required considerable technical challenges to be overcome but these reagents have been described and are soon to be commercially available.

The exquisite sensitivity of peptide–MHC tetramers for quantitating antigen-specific T cells has raised the question of whether CD8(+) cells that bind to peptide–MHC tetramers are naïve or antigen-experienced (“memory” T cells). Pittet and colleagues (18) observed that 10 of 13 melanoma patients and 6 of 10 healthy individuals had high frequencies (greater than or equal to 1/2500 CD8+ T cells) of Melan-A-specific cells in the peripheral blood. All of these Melan-A-specific cells from the healthy individuals and seven of the patients displayed a naïve CD45RA(hi)/RO(−) phenotype. In three of the patients, “memory” CD45RA(lo)/RO(+) Melan-A-specific cells were observed. In contrast, influenza matrix-specific CTLs from all individuals exhibited a CD45RA(lo)/RO(+) memory phenotype. One patient was observed to have an evolution of the Melan-A-specific cell phenotype over time. This study suggests that in addition to simply detecting peptide–MHC positive cells, it may be important to assess if antigen-specific T cells are naïve or memory T cells in order to determine if the detected antigen-specific T cells have been stimulated by the immunization strategy.

4.4. In Vitro Measures of Functional Antigen-Specific Immune Responses

In addition to phenotypic assays, in vitro assays of T-cell function play an important role in detecting and quantitating antigen-specific immune responses. Functional assays measure a T-cell activity in response to a specific antigenic stimulus, include proliferation, cytokine secretion, and cytolytic function. These assays can be performed on specimens stimulated in vitro with antigen and cytokines, or on PBMC samples, without any preceding in vitro stimulation.

4.4.1. LYMPHOPROLIFERATION ASSAY

The capacity of T cells to proliferate in response to antigen has traditionally been used as a marker of the presence of antigen-specific CD4+ helper T cells. Typically, the specimen of purified T cells or PBMCs is mixed with various dilutions of antigen or antigen in the presence of stimulator cells (irradiated autologous or HLA-matched antigen-presenting cells). After 72–120 h, [³H]-thymidine is added and DNA synthesis (as a measure of proliferation) is quantified by using a gamma counter to measure the amount of radiolabeled thymidine incorporated into the DNA. A stimulation index (SI) can be

calculated by dividing the number of counts per minute for the specimen by the number of counts per minute in cells incubated without antigen as a control.

The proliferation assay has been in use for a long time and it has frequently been used in clinical trials to compare T-cell responses before and after immunization (6,7,38–41). Depending on the immunization strategy, a small percentage (38,39) to as many as half (40) or all (41) patients have been found to respond by proliferation assays. Its major advantage is the ability to perform the assay directly on peripheral blood samples, giving a picture of the T-cell activity present *in vivo* (although the long *in vitro* culture period can introduce artifacts into the results). Its drawbacks are that it does not measure activity with direct mechanistic relevance to tumor rejection. Despite its being one of the most widely used assays, it has not yet been convincingly correlated with clinical outcome (6,38) and it can be influenced by the nonspecific immune function of the patients. The ability to proliferate does not mean that the T-cell activity induced could necessarily destroy tumor cells. And, the SI does not necessarily correlate with the number of antigen-specific T cells present *in vivo*. High levels of proliferation by a few cells or low levels of proliferation by many cells would give a similar SI. A recent flow cytometric assay measuring distribution of cell membrane dyes into daughter cells produced during proliferation permits the number of antigen-responsive cells in a stimulation assay to be determined, addressing one of these concerns (42).

4.4.2. DETECTION OF SECRETED CYTOKINES BY ELISA, ELISPOT, CYTOMETRIC BEAD-BASED ASSAYS

Cytokine secretion plays an important role in antigen-responsive T-cell activation. Consequently, one method for detecting the presence of antigen-specific T cells is by measuring either bulk cytokine production (by an enzyme-linked immunosorbant assay [ELISA]) or enumerating individual cytokine-producing T cells (by an enzyme-linked immunospot assay [ELISPOT]). In the ELISA assay, PBMC specimens are incubated with antigen (with or without antigen-presenting cells) and, after a defined period of time, the supernatant from the culture is collected and added to plates coated with antibody for cytokines of interest such as IFN- γ , tumor necrosis factor (TNF)- α , GM-CSF, or IL-2. Antibodies ultimately linked to a detectable label or reporter molecule are added and the plates are washed and analyzed. ELISA has been used for monitoring in many clinical trials (6,7,40), although the definition of a positive result differs (e.g., an amount of IFN- γ per well, which is two times greater than control wells). Because this is an assay of the cytokine production of a population of cells, it does not give information about individual cells and cannot be used to enumerate the antigen-specific T cells. Furthermore, it does not measure the actual cytokine profile of these cells *in vivo*, but rather, the ability of the cells to secrete cytokine when exposed to an antigenic stimulus *in vitro*. The ELISA assay can also be used to determine the levels of cytokines in serum or other body fluids. Although this may give a broad picture of the inflammatory state of a patient, it cannot be used to evaluate the cytokine profile in the milieu of the actual tumor cells. Flow cytometric-based assays to measure cytokine levels in culture supernatants have also been introduced and offer the capacity to measure multiple cytokines in a single sample. Up to six different cytokines can be analyzed by the commercially available Cytokine Bead Array, for example, whereas other technologies offer the capacity to measure many more cytokines in multiplex fashion using bead technologies (43,44). Increasingly, Th1 and

Th2 cytokines are being measured in cancer vaccine studies and these flow-based methods offer the capacity to perform “multiplex” analysis of multiple cytokines.

The ELISPOT assay has been refined for use in clinical trials since its initial description in 1988 (45) and it allows the number of antigen-reactive T cells to be enumerated. Although there have been several modifications depending on the immune response being monitored, the basic assay involves six steps: (a) coating a 96-well microtiter plate with purified cytokine-specific antibody, (b) blocking the plate to prevent nonspecific absorption of random proteins, (c) incubating the cytokine-secreting T cells with stimulator cells at several different dilutions, (d) lysing the cells with detergent, (e) adding a labeled second antibody, and (f) detecting the antibody–cytokine complex. The product of the final step is usually an enzyme/substrate reaction that generates a colored product representing a permanent “footprint” that can be quantitated microscopically, visually, or by electronic image analysis. Each colored spot represents one single cell secreting the cytokine of interest, provided the cell density does not exceed desirable levels (i.e., where greater than a monolayer of cells exist in the assay well). The antigen-specific T-cell precursor frequency is calculated by dividing the number of spots (cytokine-secreting cells) by the number of cells placed into the well. The ELISPOT assay has been shown to reliably detect the number of antigen-specific T cells in experiments in which known quantities of antigen-specific T cells were added to bulk PBMC preparations (46). Miyahira and colleagues have reported that the CTL precursor frequency provided by ELISPOT assay is comparable to that obtained by the limiting dilution analysis (LDA) (47). Although rigorous statistical analysis has not been performed yet, there is interest in determining whether the ELISPOT assay correlates with survival (48). In a retrospective analysis of melanoma patients immunized with a polyvalent vaccine (49), MAGE-3 and Melan-A/MART-1-specific IFN- γ -secreting T cells were counted by ELISPOT. Those patients who demonstrated antigen-specific T-cell secretion of IFN- γ had a longer recurrence-free survival (greater than 12 mo) than nonresponders (3–5 mo).

Because the task of counting the number of spots visually becomes difficult and time consuming with large numbers of spots (>100), computerized plate readers using digital cameras have been developed (50). In addition, current methods allow evaluation of a single cytokine at a time, but Okamoto (51) and colleagues have now described a dual-color method for evaluating two different cytokine release patterns at a time. This is of particular interest given that the presence of Th2-type cells that might result in secretion of suppressive cytokines is now of increasing interest in cancer immunotherapy. Using a dual-color ELISPOT, it should be possible to measure a Th1 and a Th2 cytokine simultaneously. The ELISPOT assay is also being adapted for measurement of antibody-secreting B cells (52).

4.4.3. DETECTION OF INTRACELLULAR CYTOKINES BY MULTIPARAMETER FLOW CYTOMETRY

It was first demonstrated in murine models that different patterns of cytokine secretion could be used to differentiate between memory/effector T cells with different immune functions (53). The two patterns, Th1 with secretion of IL-2, IFN- γ , and TNF- α , and Th2 with secretion of IL-4, IL-5, IL-6, IL-10, and IL-13, have also been described in humans. It is possible to monitor immune responses in humans by characterizing the cytokine secretion pattern of T cells in peripheral blood, lymph nodes, or tissues by flow cytometry

(reviewed in 54). Using flow cytometry offers a number of advantages: (a) the speed with which the assays may be performed, (b) the ability to analyze cytokine secretion on an individual cell basis while simultaneously determining other characteristics of the cell phenotype (such as CD4/CD8 expression, or memory versus naïve phenotypic markers), (c) the lack of interference with measurements by variability in the concentration of cellular or free cytokine receptors that can occur with ELISAs by reducing the cytokine content of the culture supernatant, and (d) the capacity to measure a variety of cytokines for quantitating Th1 and Th2 cytokines. Most flow cytometric methods involve a short period (4–6 h) of in vitro T-cell activation (using antigen or mitogens) and source of stimulator cells (autologous antigen-presenting cells or PBMCs). For the last 3–4 h of stimulation, cytokine secretion is prevented by the addition of brefeldin A. Following this stimulation period, the cells are fixed and permeabilized and stained with fluorochrome-conjugated anti-CD4 and anti-CD8 antibodies to allow gating on T cells, anti-CD69 to monitor activation of T cells, and an antibody to the cytokine of interest (e.g., IFN- γ , or IL-2). Three- or four-color flow cytometry is performed and the percentage of CD4(+) or CD8(+) CD69(+) cytokine(+) T cells is enumerated. This assay has been modified so that it may be performed on PBMCs (55) or whole blood (56).

The serial analysis of intracellular cytokine induction has demonstrated correlation with clinical outcome. In a phase I/II study of immunization with SRL 172 in patients with stage IV malignant melanoma, lymphocyte activation was assayed prior to each vaccine administration using a FACS-based intracellular cytokine assay (57). Induction of intracellular IL-2 production was associated with improved survival. Surprisingly, induction of IFN- γ or IL-2 plus IFN- γ was not associated with improved survival, demonstrating the complexities in choosing surrogate markers. Reinartz (58) followed intracellular cytokine production in T cells obtained at various time points during immunization of ovarian cancer patients with the anti-idiotype vaccine ACA125. Early in the immunizations, predominantly IL-2 and IFN- α were observed but later a Th2 pattern was observed. This correlated with generation of anti-anti-idiotype antibodies and prolonged survival.

4.4.4. MEASUREMENT OF CYTOKINE mRNA LEVELS BY REAL-TIME QUANTITATIVE RT-PCR

Quantitative RT-PCR is a highly accurate molecular method for measuring the levels of transcripts of a gene or genes of interest in sample RNA (59). Kruse (60) applied the technique to the analysis of cytokine mRNA from cryopreserved normal donor blood samples. Recently, Kammula (61) applied the technique in clinical trials of melanoma peptide-based vaccines to detect antigen-specific T-cell responses by comparing pre- and post-vaccine samples from melanoma patients. Peripheral blood samples and tumor tissues obtained by fine-needle aspiration (FNA) were evaluated. For PBMC samples, the method entails thawing cells into fresh medium, allowing them to recover from thawing for 10 h, and then incubating the cells for a further 2 h with either the peptide used in the vaccine or an irrelevant peptide, followed by total RNA isolation. Quantitative RT-PCR was then used to measure cytokine mRNA levels in the samples. Data were normalized to expression of a control gene, such as CD8. This study showed that quantitative RT-PCR can be used to detect antigen-specific T-cell responses in peripheral blood samples. Additionally, localization of antigen-specific T cells to tumor sites was demonstrated by analysis of FNA samples from tumor without any in vitro stimulation step. Further

studies are needed, in part to examine the relative sensitivity of this methodology, including comparative studies against other assay techniques, to determine the reliability of the methodology.

4.4.5. DIRECT CYTOTOXICITY ASSAYS

One way that T cells destroy tumors is by direct cytolysis, predominantly by CD8+ CTLs with TCR specific for 8–10 amino acid peptides derived from tumor antigen proteins and presented within the groove of the tumor MHC class I molecules on the surface of tumor cells. Thus, measurement of the ability of T cells to lyse tumor is a relevant marker for *in vivo* antitumor activity and has been used for immune monitoring in studies of passively delivered T cells (62) and active immunotherapy approaches (38,63). The microcytotoxicity assay involves mixing the specimen containing T cells or PBMCs with antigen-expressing targets loaded with Chromium-51 or Europium and measuring the release of the chromium or Europium following target cell lysis. Since autologous tumor is often difficult to obtain, surrogate targets are often employed such as HLA-matched allogeneic tumor cell lines and targets that can be loaded with the antigen of interest (such as autologous DCs loaded with peptide or genetic material encoding the antigen, or T2 cells loaded with peptide). Targets sensitive to natural killer (NK) cell lysis (K562 and Daudi) are also included to determine the level of nonspecific lytic activity. The percentage lysis of the targets after incubation for several hours is calculated by comparison to the maximum achievable lysis of the target. Using different effector cell to target cell ratios, it is possible to derive a value for the potency of cytotoxicity measured in “lytic units,” the number of T cells needed to achieve a stated amount of lysis. Theoretically, lytic units can be used to compare various CTL preparations.

One significant disadvantage to the microcytotoxicity assay is its relative insensitivity. Though bulk CTL assays represent a useful technique to give high vs low or positive vs negative readouts, they are not particularly quantitative. Furthermore, there is a need to stimulate the CTLs multiple times before testing their lytic activity (38), since it is unusual to find antigen-specific lysis by cells directly isolated from the peripheral blood even in vaccinated patients (64). These multiple stimulations likely distort the composition and activity of the T-cell population from its original state. Also, as discussed above, since autologous tumor is difficult to obtain, other targets must be used that may not reflect the actual ability to lyse autologous tumor cells *in vivo*. For example, tumor cells may downregulate their MHC molecules or upregulate their own fas-ligand causing T-cell apoptosis. It has also been shown that the CTL response is heterogeneous with different levels of avidity for the antigen (65). Since targets used for *in vitro* testing usually express high levels of antigen, lysis may not reflect the ability to lyse tumor *in vivo* if the *in situ* tumor expresses low levels of the antigen. Finally, questions as to the correlation with clinical response have been raised. In at least one study, clinical regressions were observed in two patients in the absence of CTL activity (63). Modifications to the cytotoxicity assay that make it simpler and more reproducible are being developed. Recently techniques for using flow cytometry to measure cytolysis of targets have emerged. In this assay, a cyanine membrane dye (DiO) is used to stain the target cells and an iodide nuclear dye (PI) to evaluate dead cells, providing a method of reliably differentiating target and effector cell populations. It appears that this methodology does not suffer from the high nonspecific lysis of target cells caused by ^{51}Cr labeling, which may permit analysis of targets that are notoriously difficult to label with ^{51}Cr , such as DCs and fresh tumor cells.

4.4.6. QUANTIFYING CTL PRECURSORS BY LIMITING DILUTION ANALYSIS

A cumbersome but more quantitative assay for CTL precursor frequency is LDA, which was introduced three decades ago (66). LDA analyses involve the serial dilution of T cells in a very large number of wells followed by an *in vitro* stimulation phase and target lysis phase. Poisson distribution analysis is applied to the results to determine the proportion of wells at a particular T-cell dilution that have ≥ 1 antigen-specific precursor at the start of the stimulation. Analysis of the frequencies of positive wells in successively higher dilutions as a function of \log (T cells/well) generates a line, the slope of which is proportional to the precursor frequency. In addition to being cumbersome, labor intensive, and extremely operator dependent, the LDA is also flawed by the intrinsic assumption that a single antigen-specific T cell can be expanded during the stimulation phase to generate a signal above a mathematically determined threshold.

Since the LDA assay is somewhat complicated and because a large number of cells are required for testing antigen specificity by LDA, it has been used in few published cancer vaccine studies. Moller (67) evaluated the T-cell response to immunizations of melanoma patients with IL-7 gene-modified autologous tumor cells using LDA and found that post-vaccination, PBMCs contained an increased number of tumor-reactive proliferative as well as cytolytic cells. In three of six patients, the frequencies of antimelanoma cytolytic precursor cells increased between 2.6- and 28-fold. Two of these patients showed a minor clinical response. The same group demonstrated similar results with IL-12 gene-modified melanoma cells (68). D'Souza and colleagues (69) evaluated Melan-A/Mart-1-specific CTL precursors in melanoma patients using LDA and also demonstrated that they expressed a memory phenotype. More recently Romero (70) has improved the assay methodology to increase the ability to quantify the number of precursors. Thurner (8) used this modification, which involves one cycle of *in vitro* stimulation with antigen before testing the cells for cytotoxicity, to measure MAGE-3A1 peptide-specific immune responses in a study of immunizations with DCs loaded with MAGE3A1 peptide. Eight of 11 patients were found to have increases in MAGE3A1 CTL precursor frequency after immunization. Nonetheless, it is likely that this assay will be less frequently used in the future as newer assays that are more sensitive and less cumbersome become more widely accepted.

5. COMPARISON OF THE ASSAYS

There is a dearth of studies that have directly compared the various assays for their performance in evaluating immune responses. Tan and colleagues (71) showed that estimates of CD8+ T-cell frequencies specific for EBV-related antigens varied considerably according to the method used. Values obtained from MHC-peptide tetramer staining were, on average, 4.4-fold higher than those obtained from ELISPOT assays, which were, in turn, on average, 5.3-fold higher than those obtained from LDA. Tetramer staining showed that as many as 5.5% circulating CD8+ T cells in a virus carrier were specific for a single EBV lytic protein epitope. Kuzushima (72), using EBV-specific T-cell lines, confirmed that flow cytometric analysis is more sensitive than LDA for CTL precursors and ELISA in detecting IFN- γ -producing T cells. The results of direct T-cell staining using multimeric peptide-MHC complexes raises important questions about the meaning of precursor frequencies estimated from LDA. One possible explanation for this discrepancy is that only a fraction of cloned T cells are lytic; however, functional assays

on sorted tetramer binding cells argue against this. Another major difference between the LDA and the direct-detection assays, such as tetramer staining, is that the LDA depends on cell division. Greater than 10 divisions of a single precursor would be necessary during the stimulation phase of the LDA to register as a positive response. In cases of chronic viral infection, the precursor frequencies estimated by LDA appear to be closer to those estimated by direct staining with multimeric MHC-peptide. The LDA may therefore give a more meaningful figure of T cells with long-term growth potential. Currently, several different assays are necessary until it can be established which correlate the best with clinical outcome. At this time, the assays that show most promise are the ELISPOT, intracellular cytokine staining, peptide–MHC multimer analysis. These three assays are being compared head-to-head in ongoing studies and their relative sensitivity and specificity are being determined (Hobeika et al., submitted). Indeed, the National Cancer Institute has funded an Immune Monitoring Consortium to perform studies to validate the utility of these assays for immune monitoring of cancer vaccine trials.

6. CONCLUSIONS AND AREAS FOR FURTHER INVESTIGATION

The development of assays for detecting immunologic responses to cancer vaccines is essential if these strategies are to be optimized. Standards are needed for performing the assays and interpreting the results to allow different clinical trials to be more directly compared. Agreement is needed on whether to analyze samples directly isolated from blood or lymph nodes or after a period of in vitro stimulation. Because the various assays yield estimates of antigen-specific T cells that sometimes differ in magnitude, it is critical to compare the immune response detected by a particular assay in a particular patient with the specific immune response for a well-established, immunogenic antigen, such as EBV or CMV peptide. Finally, it is important to determine what constitutes an effective level of immunologic response. For example, analysis of PBMCs from people with EBV-associated acute infectious mononucleosis using EBV peptide–MHC tetramers reveal that between 7 and 44% of circulating CD8+ T cells are EBV specific in acute infection. Similarly, in chronically infected persons, up to 2% of circulating CD8+ T cells are directed against lytic epitopes, implying continuing turnover of virus and infected epithelial cells. Whereas this level of immune response may be necessary to control EBV infections, it is not currently known what level of immunity would be sufficient to eradicate established tumor or prevent recurrence of microscopic disease in patients. Estimates from transgenic mouse experiments (73) suggest that protection from tumor challenge requires relatively small numbers of tumor-specific CTLs, in the 12–30,000 cell range, which would be in the 35–100 million range for humans based upon simple extrapolation of blood volumes, suggesting 10–30% of CD8+ T cells would need to be tumor specific. Clearly, rapidly growing mouse tumors are not comparable to a naturally arising human tumor, so these simple calculations are mere speculation; but they do suggest that levels seen in viral diseases are not an unreasonable target.

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Analysis of Humoral Immune Responses in Vaccine Trials

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CONTENTS

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1. INTRODUCTION

It has been widely recognized that both cell-mediated and humoral immunity play important roles in the inhibition of tumor growth in vaccinated cancer patients. This chapter will only briefly summarize available data that emphasize a role of cellular immunity, including proliferating, cytokine-secreting, cytolytic and/or delayed-type hypersensitive (DTH) T lymphocytes, in the control of tumor growth. We will elaborate on why T lymphocytes often are ineffective against tumors, emphasizing the important role of humoral immunity in cancer growth control. This role has recently been highlighted by the approval of monoclonal antibody (MAb) treatments for cancer patients by the Food and Drug Administration (FDA). However, the biological half-life of Mabs is short, rendering the induction of long-lasting active specific humoral immunity by vaccines a highly attractive alternative. Studies in animals have demonstrated a positive correlation between the induction of antibody responses following vaccination and tumor regression. Similarly, a correlation between humoral immune response induction and beneficial clinical responses has been demonstrated in cancer patients. Since vaccine-

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induced antibodies must bind to the tumor cell surface in order to destroy tumor cells in conjunction with effector cells or complement, emphasis is placed on those studies that have demonstrated the induction of tumor cell-binding antibodies by vaccines in cancer patients. The vast majority of studies have demonstrated binding of the elicited antibodies to proteins only and are only summarized here.

2. ROLE OF CELLULAR IMMUNITY IN THE CONTROL OF TUMOR GROWTH

Several studies suggest a beneficial role of proliferating T lymphocytes induced in vaccinated patients with melanoma (1), non-Hodgkin's lymphoma (2), and prostate carcinoma (3–9) (Table 1). These lymphocytes may have provided helper function and, therefore, may have induced cytolytic T lymphocytes (CTLs) that lysed the tumor cells. A positive correlation between CTL induction by vaccination and tumor regression and/or enhanced survival has been demonstrated in colorectal carcinoma (CRC) (5), melanoma (6), and non-Hodgkin's lymphoma (9,10) patients. Furthermore, the induction of DTH reactions in vaccinated melanoma patients has been associated with beneficial clinical outcome (7,11–14). These studies emphasize the importance of cellular immunity induction by cancer vaccines for induction of tumor regression and survival enhancement.

However, several mechanisms by which tumor cells might escape T-cell surveillance have been described:

1. Major histocompatibility complex (MHC) downregulation or mutation on tumor cells. Downregulation and/or mutation of human leukocyte antigen (HLA) class I has been demonstrated in melanoma and carcinomas of the breast, lung, head and neck, colon, cervix, and prostate (reviewed in ref. 15). This process impairs the ability of tumor cells to present antigen-derived peptide to T lymphocytes. Although reduced MHC class I expression prevents tumor recognition by CTLs, it may target the tumor cells for natural killer (NK) cell lysis (16).
2. Defects in transporters associated with antigen processing (TAPs). These defects, which have been observed in carcinomas of the head and neck, lung, colon, and cervix and in melanomas, are associated with defective processing and presentation of antigenic peptides to CTLs (reviewed in ref. 15).
3. Lack of costimulatory molecules. Costimulatory molecules, such as B7 on tumor cells, enhance the induction of tumor-specific T cells (17). However, many tumors lack costimulatory molecules, driving immune effectors into a state of anergy, unless tumor cells are cross-presented by antigen-presenting cells expressing costimulatory molecules (18).
4. T-cell inhibitory factors secreted by tumor cells. Tumor cells can secrete immune inhibitors, such as transforming growth factor- β (TGF- β), interferons, prostaglandins, and interleukins (IL)-10, which have been shown to inactivate tumor-specific T lymphocytes (19).
5. T-cell inhibiting tumor-associated molecules. The breast carcinoma-associated antigen MUC1, the ovarian and uterine cancer-associated antigen RCAS1, and the colon carcinoma-associated decoy receptor DcR3 have been shown to induce apoptosis in T cells (20–22). Similarly, Fas ligand, expressed by myeloma, melanoma, and hepatocellular carcinoma, may induce apoptosis in T lymphocytes (23). Recently, the novel T-cell inhibitory molecule B7-H1 expressed by carcinomas of the lung, ovary, and colon has been shown to actively inhibit immune responses by promoting apoptosis of effector CTLs via induction of Fas ligand and interleukin (IL)-10 (24).

Table 1
Correlation Between Immune and Clinical Responses in Vaccinated Cancer Patients

Cancer Type	Vaccine	Adjuvant	Trial Phase	Humoral Immune Response		Cellular Immune Response		Correlation Between Clinical and Immune Responses	Ref.
				No. of Positive Patients/ Total No.	Assay (Target) (Isotype)	No. of Positive Patients/ Total No.	Assay (Stimulant/ Target)		
Breast carcinoma	Sialyl-Tn-KLH	None	I	24/24	ELISA (sialyl-Tn) (IgG, IgM)	ND	ND	14/24 PR, 5/29 CR	Ab response associated with survival 50
Colorectal carcinoma	CEA-peptide-pulsed DCs	None	I	ND	ND	7/12	³ HTdR, ⁵¹ Cr-release	1/12	Association of CTL and clinical response 5
Melanoma	Melacine	DETOX	I	5/22	ELISA (melanoma cells) (IgG)	13/22	³ HTdR, ⁵¹ Cr-release	2/22 CR, 3/22 PR	CTL associated with remission 6
Melanoma	Allogeneic melanoma cell line	Alum	I	ND	ND	22/108	DTH (tumor cells)	32% increase in disease free survival	DTH response associated with disease-free survival 11
Melanoma	Anti-Id (HMW-MAA)	BCG	I	14/25	ELISA (HMW-MAA)	ND	ND	3/25 PR	Ab response associated with survival 52
Melanoma	Polyvalent melanoma vaccine (MCV)	ND	II	17/26	FACS (melanoma cell)	7/26	DTH (MCV)	3/40 CR, 6/40 PR, 4/40 SD, 27/40 PD	DTH and Ab associated with survival 12, 55
Melanoma	GM2	BCG	I	57/120	ELISA (GM2) (IgM)	ND	ND	23% increase in disease-free survival	Association between antibody titer and increase in overall survival 72
Melanoma	Autologous melanoma cells	BCG	I/II	ND	ND	19/62	DTH (autologous tumor cells)	36/62 SD	DTH associated with survival 13

Continued

Table 1 (Continued)
Correlation Between Immune and Clinical Responses in Vaccinated Cancer Patients

Cancer Type	Vaccine	Adjuvant	Trial	Phase	Humoral Immune Response		Cellular Immune Response		Correlation Between Clinical and Immune Responses	Ref.
					No. of Positive Patients/ Total No.	Assay (Target) (Isotype)	No. of Positive Patients/ Total No.	Assay (Stimulant/ Target)		
Melanoma	Polyvalent MCV	BCG	II	23/23	Western blot (Tyr, TRP1, 2 gp100)	135/283 DTH 35/42 ³ HTdR 16/33 ⁵¹ Cr-release	DTH, ³ HTdR, ⁵¹ Cr-release melanoma cell)	15–20% PR or CR	DTH and CTL associated with survival	7,8
Melanoma	Melan-A/MART peptide	IFA	I	ND	ND	ND	12/20	ELISA, IFN- γ	16/25 SD, 3/25 PD	Association of IFN- γ response with disease-free survival
Melanoma	Autologous DNP-modified melanoma cells	BCG	III	ND	ND	ND	162/284	DTH (autologous tumor cells)	36/62 SD	DTH associated with survival
Non-Hodgkin's lymphoma	Autologous tumor Ig (Id)-KLH	SAF1	I	3/9	ELISA (tumor- Id)	4/9	³ HTdR (Id)	2/9 CR	Proliferative lymphocyte response associated with clinical response	2
Non-Hodgkin's lymphoma	Autologous tumor Ig (Id)-KLH	Threonylmuramyl-dipeptide	I	5/16	ELISA (tumor- Id) (IgG, IgM)	11/16	⁵¹ Cr-release (lymphoma cells)	8/16 CR	CTL associated with clinical response	10
Non-Hodgkin's lymphoma	Autologous tumor Ig (Id)-KLH	GM-CSF	I	15/20	ELISA (tumor- Id) (IgG1)	19/20	³ HTdR, ⁵¹ Cr-release (lymphoma)	18/20 CR	Association between CTL response and survival	9
Non-Hodgkin's lymphoma	DC pulsed autologous tumor Ig (Id)-KLH	SAF1	I	15/23	ELISA (tumor- Id Ig)	15/23	⁵¹ Cr-release (lymphoma cells)	4/25 CR, 16/25 SD, 6/25 PD	Association between Ab response and clinical response	53

Ovarian carcinoma	ACA125 anti-Id	Alum	I/II	3/3	ELISA (CA125)	9/16	Cytotoxicity (CA125-expressing tumor cell line) 4/42 CR, 6/42 PR, 13/42 SD, 6/42 PD	Binding of Ab3 to Ab2 associated with survival	54
Prostate carcinoma	DC-PSM-P1 & PSM-P2	None	II	ND	ND	8/4	Cytokine ELISA (anti-CD3) 21/41 PD, 6/41 SD, 12/41 PR, 2/41 CR	Secretion of IFN- γ by PBMC associated with survival	3
Prostate carcinoma	DC/PAP	None	I	6/21	ELISA (PAP)	11/21	Proliferation 6/21 SD, 15/21 PD	PAP-specific lymphoproliferative response associated with survival	4

Ab: antibody to antigen

Ab2: Anti-idiotypic antibody

Ab3: Anti-anti-idiotypic antibody

Ag: Antigen

BCG: bacille Calmette-Guérin

CR: complete response

CTL: Cytotoxic T lymphocyte

DC: Dendritic cells

DTH: Delayed-type hypersensitivity

ELISA: Enzyme-linked immunosorbent assay

FACS: Fluorescence-activated cell sorter

Gp100: Glycoprotein 100

HMW-MAA: High-mol wt melanoma-associated antigen

3 HTdr: [3 H]thymidine

IFA: Incomplete Freund adjuvant

IFN- γ : Interferon- γ

Ig: Immunoglobulin

KLH: Keyhole limpet hemocyanin

MCV: Melanoma cell vaccine

ND: Not determined

PAP: Prostatic acid phosphatase

PD: Progressive disease

PR: Partial response

PSM: Prostate-specific membrane antigen

SD: tyrosinase

TRP: tyrosinase-related protein

Table 2
FDA-Approved MAbs for Cancer Immunotherapy

<i>Antibody</i>	<i>Target</i>	<i>Cancer Type</i>	<i>Mechanism of Action</i>	<i>Reference</i>
Rituxan (Rituximab)	CD20	Non-Hodgkin's lymphoma	ADCC	32,41,42
Herceptin	Her-2/neu	Breast cancer	ADCC	33
Mylotarg	CD33	Acute myeloid leukemia	Immunotoxin, apoptosis	34,35,43
Campath	CD52	Chronic lymphocytic leukemia	ADCC, apoptosis	36,37

6. T-cell-signaling defects. T cells in cancer patients may show defects in signaling pathways involving the ζ chain of the T-cell receptor, p56^{lck} and p59^{fyn} proteins, Zap-70, and the expression of NF- κ B, contributing to diminished T-cell activation (25–30).
7. Regulatory T cells. We have shown recently that CD4⁺/CD25⁺ regulatory T cells inhibit induction of CTLs in fresh peripheral blood mononuclear cells and proliferation of established CTLs in a colorectal cancer patient. Suppressive effects of the regulatory T cells were mediated by TGF- β (31).

Thus, various mechanisms account for tumor cell escape from T-cell surveillance, which underscores the importance of antibodies in tumor growth control.

3. MABS IN PASSIVE IMMUNOTHERAPY OF CANCER PATIENTS

Several mAbs have successfully completed phase III clinical trials and were approved by the FDA for treatment of cancer patients (Table 2). Thus, Rituxan (anti-CD20) has been approved for the treatment of non-Hodgkin's lymphoma (32), Herceptin (anti-HER-2/neu) for breast carcinoma (33), Mylotarg (anti-CD33) for acute myeloid leukemia (34,35), and Campath (anti-CD52) for chronic lymphocytic leukemia (36,37). These MAbs have significantly enhanced survival of the treated patients. The mechanism by which these antibodies may inhibit tumor growth is through antibody-dependent cell-mediated cytotoxicity (ADCC) (Rituxan [38,39]; Herceptin [33]; Campath [36]) or apoptosis (Mylotarg [40]; Campath [37]). These studies clearly demonstrate a beneficial role of antibodies in the growth control of cancer. However, the biological half-life of passively administered MAb in the blood is usually short (2–3 d for mouse MAb [41] and 5–7 d for human/mouse chimeric antibodies [42–44]), making repeated MAb administration necessary. This increases the likeliness of induction of host immune responses against the administered MAb, rendering subsequent MAb administrations less effective. In contrast, active specific immunization may induce long-lasting humoral immune responses that can be effectively boosted by repeated vaccine administration.

4. ROLE OF ACTIVE SPECIFIC HUMORAL IMMUNITY INDUCTION IN THE CONTROL OF CANCER

4.1. Correlation Between Antibody Induction and Tumor Growth Inhibition/ Increased Survival in Vaccinated Mice

Several studies in mice have demonstrated a correlation between antibody induction and tumor growth inhibition in vaccinated mice. Early studies have shown a correlation between antibody formation to Moloney sarcoma virus and regression of virus-induced sarcomas (45). Tumor growth inhibition was mediated by ADCC. Similarly, cytotoxic antibodies to feline sarcoma virus have been implicated in tumor regression of virus-induced sarcomas in dogs (46).

Anti-idiotypic antibodies binding to the antigen-combining site of antitumor antibodies may mimic tumor antigen and induce antitumor immunity (reviewed in ref. 47). By virtue of tumor antigen mimicry, anti-idiotypic antibodies may break immunological tolerance to tumor-associated antigens that are also expressed by normal tissues (47). Wikstrand et al. (48) have recently shown that anti-idiotypic antibodies mimicking mutated epidermal growth factor receptor (EGF-RvIII) can induce EGF-RvIII-specific antibodies in mice, which protect the animals from the growth of established tumors. A recombinant vaccinia virus containing the human papillomavirus E2 protein has been shown to promote tumor regression by stimulating antibody-dependent macrophage-mediated cytotoxicity (49). Augmentation of immunoglobulin (Ig)M titers to sLe(x) and GM3 after immunization with bacille Calmette-Guérin (BCG), or with tumor cells with BCG correlated with retarded tumor growth in mice, whereas increased IgG titers to sLe(x) significantly correlated with aggressive tumor growth in mice immunized with cells without adjuvants (50). The mechanism of how antibodies can accelerate tumor growth is poorly understood.

4.2. Association Between Antibody Induction and Tumor Growth Inhibition/ Increased Survival in Vaccinated Cancer Patients

Several phase I and II clinical trials have demonstrated an association between antibody induction in vaccinated cancer patients and clinical response (enhanced survival, tumor shrinkage) (Table 1). Sialyl-Tn-KLH vaccine induced IgG and IgM antibodies to the antigen in all vaccinated breast cancer patients, and antibody induction was associated with patients' survival (51). Anti-idiotypic antibodies binding to the antigen-combining site of antitumor antibodies may mimic tumor antigen. Anti-idiotypic antibody vaccine mimicking the high-molecular-weight melanoma-associated antigen (HMW-MAA) elicited anti-anti-idiotypic antibodies (Ab3; antibodies binding to anti-idiotype or Ab2) in melanoma patients, and the Ab3 responses were associated with patients' survival (52). However, in neither of the two studies were the antibodies shown to be associated with clinical responses demonstrated to bind to tumor cell-surface determinants, and, therefore, the role of these antibodies in the development of the clinical responses remains unclear. Both antibody and DTH responses induced by polyvalent melanoma cell vaccine in melanoma patients were associated with patients' survival (12). The antibodies were shown to bind to melanoma cell surfaces and may have destroyed these cells by ADCC or complement-dependent cytotoxicity (CDC) mechanisms, although this has not been directly demonstrated. In non-Hodgkin's lymphoma patients, antibody responses to

idiotype protein induced by dendritic cells pulsed with keyhole limpet hemocyanin (KLH)-coupled idiotype were associated with clinical responses (53). An anti-idiotype vaccine trial in ovarian carcinoma patients has demonstrated an association between Ab3 response and survival of the vaccinated patients (54). The Ab3 mediated ADCC against antigen-positive ovarian carcinoma cells and may be related to the beneficial clinical responses observed in the vaccinated patients.

It needs to be emphasized that most of the studies described above, with few exceptions (7,12,52,55), have shown associations, but not statistically significant correlations, between humoral immune responses and beneficial clinical responses in vaccinated cancer patients. In the clinical trials conducted by Morton and colleagues in melanoma patients (7,12,55), immune responses to allogeneic tumor cell vaccine were significantly ($p < 0.05$) correlated with patients' survival. Similarly, a statistically significant ($p < 0.0001$) correlation between anti-HMW-MAA antibodies and survival has been demonstrated in melanoma patients vaccinated with anti-idiotype (52).

Table 1 also summarizes the clinical trials that have demonstrated associations between vaccine-induced cell-mediated immunity and beneficial clinical responses. Thus, induction of CTL responses by the vaccines was associated with beneficial clinical responses in patients with CRC, melanoma, and non-Hodgkin's lymphoma (5–7,9,10), and induction of DTH and proliferative lymphocyte responses was associated with clinical outcome in patients with melanoma, non-Hodgkin's lymphoma, and prostate carcinoma (2,4,7,8,11–14,55). The correlations between cellular immune responses and clinical immune responses were statistically significant in a few studies. Thus, CRC patients vaccinated with peptide-loaded dendritic cells demonstrated a significant ($p = 0.002$) correlation between CD8+ tetramer+ T-cell responses and tumor regression (5). A statistically significant correlation ($p < 0.05$) between DTH responses and survival was demonstrated in melanoma patients vaccinated with allogeneic tumor cells (8) or autologous, hapten-modified tumor cells (13).

4.3. Assays to Determine Antibody Binding to Tumor Cells

In the following subheading, the antibody responses of vaccinated cancer patients will be discussed in detail. Emphasis is placed on those studies that determined antibody responses to tumor cells, disregarding the studies describing humoral immune response induction to tumor-derived proteins in the absence of a description of antibodies binding to tumor cell surfaces. This decision was made in light of the important role that antibodies to tumor cell–surface proteins play in tumor cell destruction via cell-mediated (ADCC) and complement-mediated (CDC) effector mechanisms. Thus, human antibodies of IgG1 isotype mediate ADCC, and IgG1, IgG2, and IgM mediate CDC (56).

Various assays have been used to detect human antibody binding to cell surfaces (57,58):

1. *Immunofluorescence assay (IFA)*. In this assay, tumor cells are incubated with human antibody, followed by incubation with fluoresceinated animal-derived antihuman Ig subclass specific antibodies or anti-F(ab)₂ antibodies. Because antihuman F(ab)₂ antibodies include an increased proportion of anti-light chain antibodies as compared to anti-Ig class/subclass-specific antibodies, antihuman F(ab)₂ antibodies have an increased chance of reacting with antibodies of various isotypes.

2. *Radioimmunoassay (RIA)*. Tumor cells are incubated with human antibody, followed by incubation with radiolabeled animal-derived antihuman Ig class/subclass-specific antibodies or antihuman F(ab)₂ antibodies.
3. *Enzyme-linked immunosorbent assay (ELISA)*. Tumor cells are incubated with human antibody, enzyme-labeled animal-derived antihuman Ig class/subclass-specific antibodies or antihuman F(ab)₂ antibodies, followed by incubation with substrate.
4. *Mixed hemadsorption assay (MHA)*. Tumor cells are incubated with human antibody. An indicator system consisting of sheep red blood cells (SRBCs) coated sequentially with mouse anti-SRBC antibody, goat antimouse IgG antibody, and mouse antihuman IgG antibody is added to the antibody-coated cells. Antibody binding to tumor cells is indicated by SRBC rosette formation around tumor cells.
5. *Immune adherence assay*. Tumor cells are incubated with human antibody. An indicator system consisting of human RBCs with C3 complement component bound to immune adherence receptor on RBCs is added. C3 will provide a bridge between RBCs and cell-bound antibody. Antibody binding to tumor cells is indicated by RBCs rosette formation around tumor cells.
6. *C3-MHA*. Tumor cells are incubated with human antibody and complement. Indicator system consisting of SRBCs coated with anti-SRBC antibody, complement, and anti-C3 antibody is added. Antibody binding to tumor cells is indicated by SRBC rosette formation around tumor cells.
7. *Protein A assay*. Tumor cells are incubated with human antibody. Indicator system consisting of human RBCs coupled with protein A is added and antibody binding to tumor cells is indicated by RBC rosette formation around tumor cells.

In all assays, except for ELISA, the percentage of antibody-positive cells as well as antibody binding intensity per cell are determined.

We have compared the sensitivity of IFA, RIA, ELISA, and MHA for detection of tumor cell-binding antibodies in the sera of CRC patients vaccinated with anti-idiotypic antibodies (57). Of the four assays, the MHA was the most sensitive and reproducible assay for the detection of serum antibodies. This assay was able to detect <0.1 µg of chimeric human × mouse anti-CRC antibody binding to tumor cells (57). To our knowledge, the sensitivities of the C3-MHA, immune adherence assay, and protein A adherence assay have not been reported.

4.4. Antibody Responses in Vaccinated Cancer Patients

Table 3 summarizes humoral immune responses in vaccinated cancer patients including only those studies in which the binding of vaccine-induced antibodies to the surfaces of tumor cells was demonstrated. Antibodies binding to the tumor cell surface may destroy the cells via ADCC or CDC mechanisms or induce apoptosis, whereas antibodies binding to intracytoplasmic structures are most likely of no benefit to the patient. Thus, there are numerous reports on the induction of antibodies to isolated tumor proteins in vaccinated cancer patients, in the absence of demonstration of antibody binding to tumor cell surfaces, and these studies are not reported here.

4.4.1. COLORECTAL CARCINOMA (CRC)

The humoral immune responses of vaccinated CRC patients are summarized in Table 3. CRC patients were immunized with anti-idiotypic antibodies (Ab2) mimicking the CRC-associated antigen CO17-1A (also termed GA733/KS1-4/KSA/EpCAM) (59–61).

Table 3
Humoral Immune Responses in Vaccinated Cancer Patients

Cancer Type	Vaccine	Adjuvant	Trial Phase	Humoral Immune Response								
				Tumor Protein Target				Tumor Cell Target				
				No. of Positive Patients/ Total No.	Assay (Target)	Specificity	Titer (Isotype)	No. of Positive Patients/ Total No.	Assay (Target)	Specificity	Titer	Ref.
Colorectal carcinoma	C017-1A anti-Id	Alum	I	8/9	ELISA (CO17-1A)	CO17-1A	>40	23/30	MHA (CRC cells)	CO17-1A	>45	59
Colorectal carcinoma	GA733 anti-Id	Alum	I	7/13	RIA (GA733)	GA733	>51	5/7	MHA (CRC cells)	GA733	>15	60
Colorectal carcinoma	CEA anti-Id	Alum	Ib	13/17	ELISA (CEA)	CEA	Low	17/23	FACS (CRC cells)	CEA	>100	62,63
								1/1	ADCC (CRC cells)	ND	ND	
Colorectal carcinoma	CO17-1A anti-Id IgG/F(ab') ₂ -KLH	Alum	I	ND	ND	ND	ND	6/23 (IgG) 9/22 [F(ab') ₂ -KLH] ²	MHA (CRC cells)	CO17-1A	>45	61
Colorectal/ pancreatic carcinoma	Recombinant GA733-2A	Alum	I	5/12	RIA (GA733-2E)	GA733-2A	>15	6/12	MHA (CRC cells)	GA733	>45	64
Melanoma	Autologous + Cy + BCG allogeneic melanoma cell lysates + VSV	I	ND	ND	ND	ND	ND	16/24	IA (melanoma cells)	Melanoma cells	>4000	65

Melanoma	Allogeneic melanoma cells	BCG or <i>C. parvum</i>	I	ND	ND	ND	ND	16/20	IA (melanoma cells)	Melanoma cells	>100000	66
Melanoma	Anti-Id (HMW-MAA)	BCG	I	3/14	ELISA (HMW-MAA)	HMW-MAA	>10,240	5/14	RIA (melanoma cells)	Melanoma cells	>64	52, 69
Melanoma	GD3 GD3-lactone GD3-amide	BCG	I	0/9 2/6 4/6	ELISA (GD3)	GD3		4/6	FACS (melanoma cells)	Melanoma cells	ND	70
Melanoma	Allogeneic melanoma cells (MCV)	BCG	II	ND	ND	ND	ND	17/26	FACS (melanoma cells)	Melanoma cells	>38	12
Melanoma	Allogeneic melanoma cells) (Cancer Vax)	BCG	II	23/23	Western blot (cell extract)	Tyr, TRP-1, 2, gp100	>640 (IgG, IgM)	16/23	FACS (melanoma cells)	TA90	>38	7,8, 67
Melanoma	GM2	BGC + Cy, QS21	I	50/58	ELISA (GM2)	GM2	>640 (IgG, IgM)	18/20	ADCC (melanoma cells)	Melanoma cells	>640	72
Melanoma	GM2	Cy, QS21	I	4/4	ELISA (GM2)	GM2	>640 (IgG, IgM)	18/20	ADCC (melanoma cells)	Melanoma cells	>640	73
Melanoma	GM-CSF-transduced melanoma cells	None	I	7/7	Western blot (cell extract)	Melanoma cells	>100 (IgG)	7/7	FACS (melanoma cells)	Melanoma cells	>100	68
Melanoma	GM2	QS21	I	34/48	ELISA (GM2)	GN2	>640 (IgG, IgM)	9/18	ADCC (melanoma cells)	Melanoma cells	>640	74

Continued

Table 3 (Continued)
Humoral Immune Responses in Vaccinated Cancer Patients

Cancer Type	Vaccine	Adjuvant	Trial Phase	Humoral Immune Response								
				Tumor Protein Target				Tumor Cell Target				
				No. of Positive Patients/ Total No.	Assay (Target)	Specificity	Titer (Isotype)	No. of Positive Patients/ Total No.	Assay (Target)	Specificity	Titer	Ref.
Melanoma	GM2-GD2	QS21	I	29/30	ELISA (GM2/GD2)	GM2 and GD2	>2560	3/30	FACS (melanoma cells)	Melanoma cells	ND	80
Melanoma	Anti-Id (GD2)	QS21	I	10/47	ELISA (GD2)	GD2	>5200 (IgG1, IgG4)	1/1	FACS (melanoma cells)	GD2	ND	81
Melanoma	GD3-KLH	QS21	I	6/6	ELISA (GD3)	GD3	<280 (IgG + IgM)	4/6	FACS (melanoma cells)	Melanoma cells	ND	71
Non-Hodgkin's lymphoma	Autologous tumor IG (Id)-KLH	SAF-1	I	3/9	ELISA (tumor-Id)	Id	>64	1/9	FACS (melanoma cells)	Autologous tumor	ND	2
Ovary carcinoma	Viral onco-lysate	None	I	ND	ND	ND	ND	8/9	FACS (ovary carcinoma cells)	Ovary carcinoma cells	ND	75
Ovary carcinoma	Viral onco-lysate	None	I	ND	ND	ND	ND	7/7	FACS (ovary carcinoma cells)	Ovary carcinoma cells	ND	76

Ovary carcinoma	L ^y	QS21	I	16/24	ELISA (L ^y ceramide or L ^y mucin)	L ^y cera-mide and L ^y mucin	>120	8/24	FACS (ovary carcinoma cells)	Ovary carcinoma cells	ND	77
Prostate carcinoma	Globo H hexasaccharide-KLH	QS21	I	19/19	ELISA (IgM, IgG)	Globo H	>20,480 (IgM, IgG1, IgG3)	10/18	FACS (breast cancer cells)	Breast cancer cells	ND	78
Renal cell carcinoma	Autologous tumor cells	<i>C. parvum</i>	I	ND	ND	ND	ND	4/11	IA	Autologous tumor cells	ND	79

ADCC: Antibody-dependent cellular cytotoxicity

Anti-Id: Anti-idiotype

BCG: bacille Calmette-Guérin

CEA: Carcinoembryonic antigen

C. Parvum: *Corynebacterium parvum*

CRC: Colorectal carcinoma

Cy: Cyclophosphamide

ELISA: Enzyme-linked immunosorbent assay

FACS: Fluorescence-activated cell sorter

Gp100: Glycoprotein 100

HMW-MAA: High-mol wt melanoma-associated antigen

IA: Immune adherence

Ig: Immunoglobulin

KLH: Keyhole limpet hemocyanin

MCV: Melanoma cell vaccine

MHA: Mixed hemadsorption assay

ND: Not determined

RIA: Radioimmunoassay

Tyr: Tyrosinase

TRP: Tyrosinase-related protein

VSV: Vessicular stomatitis virus

or carcinoembryonic antigen (CEA) (62,63). The Ab2 induced anti-anti-idiotypic antibodies (Ab3), which bound to CRC cells (59–63) and mediated ADCC with human effector cells (62,63). Interestingly, the extracellular domain of the CO17-1A/GA733 protein was more immunogenic than Ab2 in cancer patients (64), probably a reflection of the increased number of immunogenic epitopes expressed by the extracellular domain protein as compared to Ab2, which mimics only a single epitope.

4.4.2. MELANOMA

The humoral immune responses of vaccinated melanoma patients are summarized in Table 3. Melanoma patients vaccinated with irradiated or lysed allogeneic tumor cells (7,8,12,65–67) mounted humoral immune responses to melanoma cells. Granulocyte-macrophage colony-stimulating factor (GM-CSF) transduced melanoma cells were immunogenic in all patients vaccinated and elicited tumor cell-binding antibodies (68). Immunizations with Ab2 (52,69) or ganglioside GD3 (70,71) also elicited melanoma cell-surface-reactive antibodies in patients. It is unclear whether the antibodies elicited in the vaccinated melanoma patients in the above-described trials were cytotoxic. GM2 ganglioside vaccine has been extensively studied in phase II clinical trials and has been shown to induce cytotoxic antibodies reactive in ADCC (72–74). However, this vaccine has recently failed to prove superior to interferon- α in a phase III randomized control trial.

4.4.3. OVARY CARCINOMA

The humoral immune responses of ovary carcinoma patients vaccinated with viral oncolysates or Lewis^y (L^y) antigen are summarized in Table 3. Both types of vaccines elicited antibodies binding to tumor cell surfaces (75–77), but the cytotoxic reactivities of the antibodies have not been determined.

4.4.4. NON-HODGKIN'S LYMPHOMA, PROSTATE, AND RENAL CELL CARCINOMA

The humoral immune responses of non-Hodgkin's lymphoma, prostate and renal cell carcinoma patients are summarized in Table 3. Non-Hodgkin's lymphoma patients were vaccinated with lymphoma idiotype coupled to the immunogenic carrier KLH (2). Only one of nine patients raised antibodies that bound to the surface of lymphoma cells, although antibodies binding to idiotype protein were found in three patients. Prostate carcinoma patients vaccinated with Globo H hexasaccharide coupled to KLH developed antibodies binding to breast carcinoma cell lines (78). Renal cell carcinoma patients vaccinated with autologous irradiated tumor cells developed antibodies reactive to the surfaces of autologous tumor cells (79).

5. CONCLUSIONS

Both tumor cells and T cells have developed various mechanisms that allow tumor cells to escape immune surveillance by T cells. These mechanisms do not impair the cell-binding and lytic properties of antibodies. Thus, antibodies constitute important mediators of tumor cell destruction. Attempts to induce antibodies by active immunization with tumor cell and tumor antigen-derived vaccines in cancer patients must be reviewed critically in light of the important role that antibodies binding to tumor cell surfaces play in tumor destruction. A few studies have demonstrated a statistically significant correlation between the induction of tumor-cell-surface-reactive antibodies and patients' sur-

vival or beneficial antitumor responses. Although these studies are encouraging, the role of vaccine-induced antibodies in patients' survival must be evaluated in randomized controlled phase III trials. It is possible that the optimal vaccine must induce both humoral and cellular immunity as activation of both arms of the immune system has been correlated with enhanced patients' survival. Whereas the first phase III randomized trial with a humoral immunity-inducing vaccine did not hold promise, several other phase III trials with vaccines that predominantly induce either humoral or cellular immunity or both are currently in progress. The outcome of these trials will be evaluated during the following 5 yr or sooner.

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1. INTRODUCTION

The progress made over the last few decades in understanding the genetic and immunological aspects of cancer has led to the development of a large number and variety of immune-based approaches to combat the disease. Among the products developed, or under development, have been receptor-targeted monoclonal antibodies (e.g., trastuzumab, tositumomab), cytokines (e.g., interferon- α [IFN- α], interleukin-2), growth factors (e.g., granulocyte-macrophage colony-stimulating factor [GM-CSF], granulocyte colony-stimulating factor [G-CSF], erythropoietin), and an amalgam of innovative products collectively referred to as tumor vaccines.

In the Center for Biologics Evaluation and Research (CBER) at the United States Food and Drug Administration (FDA), we are seeking to promote public health by encouraging the development of these exciting new therapies within the context of sound regulatory policy that will ensure patient safety. As tumor vaccines are a diverse and evolving set of products, the regulatory issues surrounding these products are not static. This chapter will outline some of the current regulatory issues for tumor vaccines.

*The opinions of the authors do not necessarily reflect those of the Food and Drug Administration.

Tumor vaccine products are reviewed in CBER by a team of regulatory scientists and clinicians. This team includes experts in the following fields: biologics manufacturing, processing, and characterization (the “Product Reviewer”); preclinical and clinical pharmacology and toxicology (the “Pharm/Tox Reviewer”); and clinical oncology (the “Clinical Reviewer”). Although there is some overlap in regulatory issues of these fields, for purposes of organization for this chapter the issues will be categorized by the three review functions.

2. PRODUCT ISSUES

Tumor vaccine products can generally be divided into four broad categories: cellular tumor vaccines, multiantigen preparations and tumor lysates, recombinant or purified proteins and peptides, and viral or plasmid vectors. Issues associated with viral/plasmid vector-based vaccines have a unique regulatory history that has evolved over the last 10 yr and will not be discussed in this chapter. We refer the reader to specific FDA guidance for this class of product (*Guidance for Industry: Guidance for Human Somatic Cell Therapy and Gene Therapy*, 1998). Despite the variety of products contained within these categories, several general regulatory principles apply to all of them. In this section we will discuss these general tumor vaccine product issues, and then we will describe in greater detail some of the promise and challenges that face many of the individual products.

2.1. General Regulatory Issues Related to Tumor Vaccines

Just as it is important to find novel approaches for cancer therapy, it is also important that these tumor vaccine products are developed to best reveal their therapeutic potential. The role of the product reviewer is to ensure that patients are receiving a product that is safe, pure, and potent at all stages of development. A safe, pure, and potent product is most likely to be produced if there is quality and consistency in both the testing and manufacture of the product. Although the standards outlined as Good Manufacturing Practices (GMPs) will not be achieved before licensure, many of the principles should be applied to product testing and manufacturing at all stages of product development.

2.1.1. PRODUCT MANUFACTURE

Consistent production of tumor vaccine products, particularly multiantigen preparations, autologous tumor cell lysates, and cellular vaccines is critical in order to verify and confirm any promising activities observed at the early stages of the clinical trial. Prior to a product sponsor’s submission of an Investigational New Drug (IND) application to the FDA, the product will often have already undergone pilot production/manufacturing efforts. The goal of this phase of product development is to manufacture a tumor vaccine of a quality and consistency acceptable for first use in humans in the phase I clinical trial setting. As product development continues into later stages, the quality and consistency of the tumor vaccine production should improve. Controls should be in place to ensure that the materials and methods used in manufacturing are suitable for producing a clinical-grade product. In addition, proper facilities design and maintenance as well as personnel training and oversight will contribute to the production of a safe and consistent product.

If the tumor vaccine is shipped, the shipping conditions must be shown not to have negative effects on the quality of the product. As with all biological products, a stability

program for tumor vaccines must also be established, particularly if the tumor vaccine will be stored and used for repeat administration to patients. It is important to know whether the vaccine injected initially is the same as that injected during later cycles of immunization. A stability program should be initiated by phase I trials and data acquisition should continue through phase III trials to support the dating period.

2.1.2. PRODUCT TESTING

In-process and lot-release testing are critical controls in the manufacturing process. Product testing should include testing for safety, identity, purity, and potency. Test methods should be chosen to allow for accurate and reproducible results. Some test methods are standard, such as the sterility testing outlined in the Code of Federal Regulations (21 CFR 610.12), whereas others are product-specific and are determined on a product-to-product basis, such as potency testing. Wherever standard tests cannot be used, alternate tests may be used if demonstrated to be of equal or greater sensitivity and specificity as the recommended methods.

Safety testing should include testing for sterility—including bacteria, fungi, and mycoplasma—and for adventitious viruses. Adventitious agent testing is especially important when allogeneic cells or cell lines are used in the manufacture or the final formulation of the tumor vaccine. Whenever cells or cell lines are cultured, the bulk harvest should be tested for mycoplasma because of the risk of contamination by fetal calf serum, other animal-derived reagents, or the individual(s) handling the cells. Additional information on cell-line testing can be found in the FDA document, “Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals” (1993). Identity testing should identify and distinguish the final vaccine product from other products produced in the same facility. Purity testing should ensure that harmful substances are not introduced during the manufacturing process and includes testing for endotoxin and harmful residual solvents. Determining an appropriate assay for potency testing is one of the most difficult challenges for tumor vaccines and therefore warrants further discussion.

Potency is one of the most important measures of quality for each tumor vaccine lot and should also demonstrate manufacturing consistency between lots. The establishment of a uniform potency assay for all classes of tumor vaccines remains one of the most vexing problems and impediments to the future licensure of this class of biologics. The federal regulations are clear regarding the need for a potency assay: The definition of potency as stated in 21 CFR 600.3 is “the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to affect a given result.” However, defining acceptable methods to measure potency of tumor vaccines is controversial because of the uncertainty regarding appropriate laboratory tests and the inconsistent demonstration of clinical efficacy. Nevertheless, we will provide some recommendations in the sections below. There may also be assays that can be used as surrogates for potency and can be performed for lot release. For example, potency of autologous, cellular tumor vaccines could be determined by a defined process, cell viability, and the expression of phenotypic markers known to correlate with biological activity. We anticipate that advances in the field of immunology together with advances in the techniques utilized to measure tumor identity and immunological function (e.g., genomics and proteomics) will greatly improve our ability to effectively measure potency in the future.

2.2. Tumor Vaccine-Specific Regulatory Issues

As mentioned earlier, this section is focused on three broad classes of tumor vaccines: (a) cellular tumor vaccines, (b) multiantigen preparations and tumor lysates, and (c) recombinant or purified proteins and peptides. This section will discuss regulatory issues specific to products in each of these product classes. For each tumor vaccine discussed we will provide a description, examples, current clinical applications, and some of the regulatory issues unique to that vaccine product. This is not meant to be an inclusive discussion, but an attempt will be made to cover many of the types of products currently being investigated.

2.2.1. CELLULAR TUMOR VACCINES

Cellular tumor vaccines are composed of autologous or allogeneic tumor cells, tumor-specific lymphocytes, and antigen-presenting cells (APCs). These cells can be used alone or in combination with other types of tumor vaccines. They can also be administered with adjuvant or cytokines to enhance their potential as a vaccine product.

2.2.1.1. AUTOLOGOUS TUMOR CELLS. Autologous tumor cells are derived from the patient's own tumor. These cells may be modified *in vitro* before they are injected back into patients with or without adjuvants. In some cases these tumor cells are modified by chemicals, irradiation, or by photochemical treatment in conjunction with ultraviolet (UV) light exposure. These modifications usually render the tumor cells replication incompetent, but certain modifications may enhance immunogenicity as well. It is hypothesized that these modified tumor cells will boost an immune response against tumor antigens and result in rejection of established tumors or development of immune memory that will not allow tumors to recur in cases where tumors were previously resected. Autologous tumor cells have already been used in a number of clinical trials for melanoma, colorectal cancer, ovarian cancer, and renal cancer with moderate success (1).

This kind of tumor vaccine poses unique regulatory issues in clinical development in part because each product is unique to the patient from whom it was derived, making every patient's tumor cells an individual product lot. Therefore, it is difficult to establish consistency between lots. Novel and unique approaches are needed to establish lot-to-lot consistency. The process of generating single-cell suspensions from tumor tissue may provide some consistency and must be carefully described. As tumor cells are obtained by digesting tumor tissues with different enzymes under different conditions, it is critical to determine viability of these cells and to set acceptable specifications to ensure consistency of the product. In addition, solid tumor nodules may have infiltrating cellular components; therefore, characterization of cell types in a tumor suspension is recommended.

Potency assessments are also complicated by the unique nature of each lot as well as the limited time between tumor harvest and patient administration. Nevertheless, efforts must be made to determine the type of biological response these products induce in the patient and to develop an appropriate potency assay. As mentioned earlier, demonstration of a correlation between the proportion of cell types and cell viability in the vaccine preparation with a reproducible immunological response in patients may be an approach for justifying a surrogate potency assay.

Sterility can also be a unique challenge for some autologous tumor vaccines. It can be difficult to obtain sterile tumor cells from certain visceral or subcutaneous tumors. How-

ever, investigators have been able to obtain sterile cells from these sites and sterility of these products is critical for patient safety.

Tumorigenicity is another important consideration for any vaccine containing tumor cells. Measures need to be established to prevent new tumor growth resulting from vaccine administration. Modifications such as UV or gamma irradiation can be used to render tumor cells replication incompetent; however, it is important that timing, procedures, and dose of these modifications are sufficient for that purpose.

2.2.1.2. ALLOGENEIC TUMOR CELLS. A second type of cellular tumor vaccine is allogeneic tumor cells. In contrast to many autologous tumor cell vaccines, most allogeneic tumor vaccines are not patient-specific, and are prepared and stored frozen, thus allowing additional time for characterization of the product. Donor screening and cell-line characterization are fundamental issues with allogeneic tumor vaccines. Whether cell lines are obtained from a commercial source, nonprofit organizations, or derived from an independent laboratory, the history of the cell line must be thoroughly documented and the cell line fully characterized for freedom from adventitious agents such as viruses, bacteria, fungi, and mycoplasma as described in the “Points to Consider” document available from the FDA, referred to earlier in subheading 2.1.2.

For mixed allogeneic tumor cell vaccines composed of multiple cell lines, quantitative assessment should be made to determine the identity and presence of each individual cell line. This test should be in place before initiating a phase III program. Identity assessment may be performed using various analytical methods including quantitative expression of two or three known tumor antigens uniquely present on individual cell lines. In addition, an assay to measure potency of the product should be established before initiating phase III studies. Various tests can be performed to determine potency of this class of vaccine. For example, one can assess allogeneic T-cell proliferative responses or cytokine release by responder cells.

2.2.1.3. ANTIGEN-PRESENTING CELLS. Antigen-presenting cells, such as dendritic cells or dendritic cell–tumor cell fusions, compose the third type of cellular tumor vaccines. These cells serve as an adjuvant in the immunotherapy of cancer. Dendritic cells are typically “pulsed” with tumor antigens (peptide epitopes, tumor cell lysates, or shed tumor antigens), or transfected to express such antigens. These cells have shown promising results both in animals and in humans (2,3). Dendritic cells are typically derived from autologous sources (such as peripheral blood monocytes or bone marrow-derived precursor cells) and their phenotype may vary depending on the cell source (4,5). Most commonly dendritic cells are generated *in vitro* by culturing peripheral blood monocytes in GM-CSF and interleukin-4 (IL-4). Recent studies have shown that *in vivo* administration of Flt-3-ligand may enrich dendritic cells in the blood circulation (5). IL-4 and GM-CSF activation are known to generate immature dendritic cells, whereas inclusion of other factors such as IFN- α , tumor necrosis factor (TNF)- α , CD40L, and prostaglandin causes maturation of dendritic cells. The phenotype of cells may vary depending upon the factor used to activate and mature dendritic cells. Therefore, phenotypic characterization of these cells is important for the characterization of dendritic cells. At least two or three phenotypic markers may be considered to define the identity of immature and mature dendritic cells.

Another important issue associated with this class of tumor vaccine is the potency assay, which is determined by antigen-presenting capacity, and the ability to induce an immune response as measured by proliferation of responder cells, generation of a cyto-

toxic T lymphocyte (CTL) response, or production of cytokines. Before undertaking phase III studies, the potency assay should be finalized to decide whether antigen-pulsed dendritic cell vaccines are actually activated and presenting antigen. Similar to autologous tumor cell vaccine products, autologous dendritic cells also face difficulty in determining potency prior to product administration to patients. In addition, as each dendritic cell product is unique to the patient from whom it was derived, it may have inherent differences in certain biological activities. Identification and expression of common surface markers on dendritic cells and correlation of marker(s) expression with in vitro biological response such as proliferation of allogeneic or autologous T-cell clones may serve as a potency test, which could be performed prior to patient administration of cells and for lot-to-lot consistency of products. Other correlations may be acceptable and may be developed as an appropriate surrogate potency assay.

2.2.1.4. TUMOR-SPECIFIC LYMPHOCYTES. Tumor-specific lymphocytes are the fourth type of cellular tumor vaccines. Included in this type of vaccine are autologous tumor-infiltrating lymphocytes (TILs; predominantly CD8+ T cells derived from solid tumors), antigen-specific CTLs, natural killer (NK) cells, and lymphokine-activated killer (LAK) cells. These cells are expected to be highly reactive to tumor antigens and sustain immunity against tumors. By adoptive transfer, these cells are expected to overcome tolerogenic mechanisms and preferentially proliferate and kill tumor cells. Regarding TILs, despite in vitro killing of tumor cells by these highly reactive T cells, clinical results have been disappointing. These cells generally failed to engraft and persist once adoptively transferred to hosts. However, a recent study has demonstrated that lymphodepletion of the host can increase the engraftment and survival of the administered cells and thus result in increased antitumor activity (6).

Characterization of this class of tumor vaccine product may be straightforward. Identity can be defined by phenotypic expression of CD8 or other known surface markers. If there is a combination of cells, testing should include an identification of each cell type present. Since these cells are expected to have high lytic capacity toward cells bearing tumor antigen, cytolytic activity or related responses could form the basis of a potency test.

2.2.2. MULTIAntigen PREPARATIONS AND TUMOR-CELL LYSATES

As cancer is a heterogeneous disease and cellular populations within a tumor are heterogeneous, it is hypothesized that they may express different tumor antigens, which may or may not be shared among various tumor cells within the same tumor or between tumors (7–9). Therefore it is believed that vaccination of host with multiantigen preparations may generate a robust immune response to cause complete rejection of tumor or protection from recurrence of tumor with a different antigen repertoire (10,11). These multiantigen preparations are often derived from autologous or allogeneic tumor cells, by either collection of tumor-secreted or shed proteins or lysis of cells by different procedures.

Because of the “dirty” nature of these products, multiple concerns need to be addressed to ensure lot-to-lot consistency in terms of composition of components and stability of components upon storage. For this class of cell-free tumor vaccine, the manufacturing process used to generate cell lysates or shed soluble tumor antigens and the characterization of cell source is a critical element in vaccine development. For vaccines prepared from autologous tumors, the characterization of initial cellular composition is important

as tumor cells may be obtained by digesting tumor tissues with enzymes under different conditions and thus cellular composition may differ with different cell substrate lots. Wherever possible, the composition of initial cellular components should be kept similar in all lots for the same donor or between donors. Important considerations for this class of tumor vaccines include viability of tumor cells, enzymes used to release cells from tumor nodules, determination of infiltrating immune cellular components, sterility of cells, and residual enzymes or other media components in the final vaccine lots. Mycoplasma testing at the cellular stage should be incorporated in the characterization of cell substrate.

If allogeneic tumor cells are used for acellular vaccine preparations, such cell lines will also need to be thoroughly characterized. Like the whole-cell tumor vaccines, tumor vaccines derived from cell lines should be shown to be free of adventitious agents such as viruses, bacteria, fungi, and mycoplasma. If multiple cell lines are pooled to form the product, these cell lines should be individually characterized and frozen and cells or product later mixed in defined proportion for final characterization and vaccination.

For the identity of this class of product, it is important to quantitatively define the known tumor antigens in the lysates or antigen mixture. The characterization of tumor cell lysates and multiantigen preparations can be performed by a number of available techniques. For example, total protein concentration, SDS-PAGE including Western blot analysis for known proteins or peptides, 2D-gel electrophoretic patterns, and gel filtration patterns may be performed to demonstrate lot-to-lot consistency. Suitable test methods must be chosen for each product. At least two to three known proteins/peptides in the vaccine preparations should be quantified for identity of the final product.

The potency test for this product must also be developed during early stages of the clinical program. Similar to other products, initiation of a phase III trial will depend on a well-established potency assay. Several tests can be considered for the potency assay, for example, generation of an allogeneic CTL response after pulsing with known antigens in the preparation, or T-cell proliferation or antibody responses in an animal model.

2.2.3. RECOMBINANT OR PURIFIED PROTEINS OR PEPTIDE

Unlike the multiantigen preparations and tumor lysates, recombinant or purified proteins and peptides are usually well-defined tumor antigens that are known to have selective or abundant tumor expression. In addition, these products have often been shown to be targets for the immune system. A large number of tumor-specific antigens have been identified including developmental antigens, differentiation antigens, mutant or overexpressed self-antigens, and viral antigens (12). In the clinic, these tumor-specific antigens have been used both alone and in combination with APCs, cytokines, or adjuvant. Although these products have shown limited efficacy, new approaches are being developed to augment their potential including increasing their binding affinity to the major histocompatibility complex (MHC) or the T-cell receptor (TCR), administering multiple antigens, or linking peptides together to form polypeptides.

Recombinant or purified protein and peptide tumor vaccine preparations can be more extensively characterized than other tumor vaccine products. Many analytical methods are available to characterize the identity and purity of these products. For proteins, these methods may include amino terminal sequence analysis, SDS-PAGE, tryptic digest, or high-performance liquid chromatography (HPLC). For synthetic peptides (including ganglioside, GD2, GD3, GM1, and GM2), methods may include spectrophotometric

analysis, infrared, nuclear magnetic resonance (NMR), mass spectroscopy, HPLC, and high-performance capillary electrophoresis. With many peptides, the complete sequence analysis can be used to establish the identity and purity. Organic solvents such as chloroform and acetonitrile are commonly used to synthesize and purify peptides. Verification of removal of organic solvents for safety is critical for this class of compounds before embarking on clinical studies.

The potency of this class of products could be assessed by generation of a CTL response, cytokine secretion, or proliferation assays in response to cells pulsed with the antigen. If peptides are used as vaccines alone or mixed with adjuvants or keyhole limpet hemocyanin (KLH), the potency of this class of tumor vaccine should be based on the peptides alone and with the adjuvant; or if the peptides are used to pulse the dendritic cells, the potency should be determined on the pulsed cells.

3. PRECLINICAL ISSUES

The role of the pharmacology/toxicology reviewer on the FDA regulatory review team is to assess the results of tumor vaccine efficacy experiments and toxicology studies in vitro and in animals and to help assess pharmacologic/immunogenicity measures of clinical activity in early phase clinical studies. Regulatory issues involving toxicity assessment of tumor vaccines will be discussed initially, followed by a discussion of clinical pharmacology issues such as the development of immune system surrogate markers of tumor vaccine clinical activity.

In any product development plan, the goals of the pharmacology/toxicology program are to attempt to determine the optimal initial dose and regimen (e.g., dose interval, route of administration) in human studies, and to predict the potential toxicity of the product in humans (e.g., target organs for toxicity and potential at-risk populations). Although the pharmacology/toxicology program should be tailored to the specific vaccine dose, pre-clinical evaluation will in general, comprise:

1. A description of the mechanism of action of the product, including identification of antigen type and expression profile in malignant and normal human tissues, the role of human leukocyte antigen (HLA) specificity and a description of the relevant immunologic mechanisms for antigen presentation, processing, and immune response (i.e., humoral and/or cellular pathways). In order to assess potential for organ-specific toxicity, and provide bridging data from the chosen animal toxicology model to human safety testing, information on the distribution of the target antigen in the chosen animal species should be assessed and provided with the IND submission.

Note that in some cases the mechanism of action of the product might guide aspects of the toxicology program. An active tumor vaccine product might be expected to involve cellular, humoral, and/or complement activation, so the better the mechanism has been elucidated in animals, the more likely appropriate serologic and immunologic parameters can be developed for monitoring safety and efficacy of the product in humans.

2. Animal “proof of concept” experiments to show efficacy and optimal dose of the product. These would include study of the immunogenicity of the product, absorption, distribution, metabolism, and excretion studies (appropriate for viral vector-based studies, but not generally necessary for other cancer vaccine candidates), and cancer prevention and

treatment studies using animal tumor models (e.g., human tumor xenograft models in severe combined immunodeficiency [SCID] or nude mice, or p53 mouse models).

3. Acute and chronic exposure studies to ascertain the potential toxicity of the product in the clinical trial setting. The animal model should be as predictive of potential human toxicity as possible, and dose, number of doses, and length of dosing should exceed that anticipated in the initial clinical study. These tests can, for many cancer vaccines, be used to establish the following common measures of animal dosing and toxicity for extrapolation to initial human studies: (a) NOAEL (or no adverse effect level—the immediate dose below that producing an adverse event), (b) PEL (or pharmacologic effect level—the lowest dose producing a pharmacologic effect that is different from control, but without adverse effect), (c) EF (or equivalence factor—that number used to adjust the preclinical data to be more relevant to the clinical situation, e.g., adjustments for relative potency or surface area), (d) DI (or duration index—the duration of the longest study in animals considered a reasonable approximation to the clinical study divided by the anticipated duration of the clinical study), and (e) TSF (or tentative safety factor—the NOAEL or PEL divided by the initial clinical dose).

Generally, single- and repeat-dose toxicology studies are performed in two relevant mammalian species (one nonrodent) to help identify organ-specific toxicities as per published federal guidelines (International Conference on Harmonization M3 Document: Non-Clinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals). As mentioned, the dose intensity should be greater than or equal to that anticipated in the clinical trials. As a practical matter, a high “therapeutic index” in an appropriate animal model can reassure a regulatory reviewer about the potential for lack of significant initial toxicity of a new product entering clinical trials. Included in any program where toxicity is observed would be an attempt to characterize the recovery from the toxic insult. In some cases, it might be appropriate to consider late toxicities (e.g., for viral vector-based gene therapies, cellular tumor vaccines).

Exceptions to the requirement for acute and chronic animal exposure studies occur when it is impossible to find appropriate animal models for the assessment of efficacy and/or toxicity. This latter situation is often the modus operandi for preclinical assessment of tumor vaccines. In this scenario, information about the antigen, construct, etc., and its tissue distribution and expression are critical and will be used to support the nonclinical safety assessment. In addition, there could be cases (e.g., gene therapy-based tumor vaccines) where carcinogenicity, genotoxicity, reproduction and developmental toxicology studies will be expected, but usually not as a requirement prior to initiation of phase I.

Several novel toxicities have been observed in tumor vaccine trials. These include local tolerance, autoimmunity, hypersensitivity, and the cytokine release syndrome. Therefore, the clinical trial-monitoring program should be appropriately designed to address these untoward reactions (e.g., with appropriate stopping rules, dose escalation, and monitoring for signs and symptoms in phase I trials) and an action plan should be in place before initiating clinical studies.

Finally, limitations to tumor vaccine pharmacology/toxicology programs include the inability to denote variations in the immunophysiology between the preclinical species chosen and humans. This limitation applies to species differences for the tumor tissues

under study. Other limitations include the lack of animal models of long-term toxicity of tumor vaccines, as well as models to assess the reversibility of toxicity.

3.1. Clinical Pharmacology

One goal of the clinical pharmacology program for a tumor vaccine is to identify appropriate serologic and/or immunologic parameters for monitoring clinical activity (as defined in subheading 4) of the product in cancer patients.

A consortium of laboratories has recently been formed in an effort to organize and standardize approaches for clinical immune monitoring of tumor vaccines. At the 2001 Society of Biological Therapy Conference, this consortium convened a one day workshop to carefully examine various methods of immunological monitoring for clinical activity in tumor vaccine trials (13). The following assays were recommended for clinical trial immune system monitoring: ELISPOT, CFC, tetramer, antibody, PCR, and TCR dysfunction. Among all available technologies that were evaluated, the most notable and practical approaches were identified to be the ELISPOT and tetramer assays. These assays were more quantitative and provided a clearer understanding of how the immune system reacts to vaccines and could be developed to help establish parameters for monitoring during clinical trials of tumor vaccine products. Various clinical trial–monitoring approaches were assessed and consensus recommendations were made and are summarized below for the following vaccine scenarios:

1. Monitoring of T-cell responses against defined CD8 T-cell epitopes: Recommendations were to use a combination of the ELISPOT and tetramer assays.
2. Monitoring of a response against a specific protein: Regarding this type of vaccine, the consensus was that antibody response and CD4 and CD8 T-cell response might be involved after administration. To assess antibody response, it was recommended that quantitative and specific antibody assays be adapted; e.g., ELISA to (a) measure the polyclonal antibody response to the tumor antigen, (b) identify the antibody class and IgG isotype involved in the initiation of the response, and (c) determine antibody avidity via antibody dissociation methods. In addition, antibody responses detected by ELISA are typically validated through direct visualization via Western blot analysis. Regarding assessment of CD4 response, proliferation assays have traditionally been used. However, as noted in the consensus document, analysis of CD4 response via adapted ELISPOT and CFC assays is being performed with increasing frequency.
3. Monitoring of responses against partially undefined antigens (e.g., vaccination with modified tumor cells, tumor cell lysates, or dendritic cell/tumor cell fusion products). Recommendations were to characterize a limited number of antigens discernible in the product and characterize the antibody, CD4, and/or CD8 response as described above.

Once immunological markers become well defined for each tumor vaccine class, reproducibility will be very important in order for a particular assay to make its way into general use in the clinical trial setting. Regulatory expectations include adherence to standard operating procedures and assay validation. Specific issues involved with assay validation for tumor vaccines have been recently outlined (13). For T-cell-based methods of immunologic monitoring, tumor vaccine sponsors have often not consistently applied these principles in their product development. Reasons for this inconsistency reflect the evolving knowledge still emerging about reliable assessment and monitoring of T-cell function as outlined above.

One effort under way to help in enhancing reproducibility of results in tumor vaccine clinical trials is the use of cooperative groups in the phase II setting to follow common procedures (obtaining, handling, and processing of samples, for example) and the use of centralized labs with established standard operating procedures (SOPs) for some of the more technically difficult assays. Over time, the goal is that the most promising immune biomarkers will indicate predictive potential for vaccine activity using established cancer treatment or prevention outcome measures (*see subheading 4*) and therefore allow true surrogate markers of tumor vaccine efficacy to emerge.

Another technological advance that is sure to change the manner in which all pharmaceuticals are evaluated is the emerging field of genomics and proteomics. It is deemed that these assay systems will be useful in the discovery and identification of targets, in understanding disease pathways, and in a new therapy's mechanism of action. Furthermore, these tests may allow identification of "bridging biomarkers" that might be used to monitor key damage responses in laboratory animal models and humans and may identify polymorphisms that may modify sensitivity to disease or new treatments. Whether microarray and proteomic technology will be genuinely applicable in the setting of complex biologic systems and immune responses that are the basis for tumor vaccinology remains to be seen. To be useful as development tools in evaluation of tumor vaccines, from either the safety or efficacy standpoint, the relationship of chosen microarray or proteomic endpoints with accepted clinical end points will need to be established, reproduced, and validated.

4. CLINICAL ISSUES

Tumor vaccines as a product class differ from cytotoxic agents on multiple levels. The general perception is that these products will have relatively little toxicity and will have more targeted activity against tumor tissues as compared to traditional chemotherapeutic agents. At the same time, the activity of vaccines may be subtler than the powerful acute systemic effects often observed with chemotherapy administration. Because of this, discussion inside and outside the FDA has focused on what aspects of the developmental process can or should be handled with some flexibility compared to traditional models. A public meeting organized by the FDA and the National Cancer Institute (NCI) offered various opinions on these issues (FDA/NCI Workshop on Tumor Vaccines, Bethesda, MD, December 10, 1998).

4.1. Phase I

4.1.1. PATIENT POPULATION

A common perception about the utility of tumor vaccines is that they may not optimally perform in the setting of extensive disease. Products with subtle yet specific biological activity may be overwhelmed in the complex biological and immunological setting of advanced cancer. Yet typically, because cancer patients usually have many active chemotherapy options, and the FDA is obliged to allow trials of an investigational agent only in a setting of clinical equipoise, patients enrolled in phase I trials will often have exhausted all other options and stand to gain little from a vaccine approach. Ideally efforts should be made to test tumor vaccines in immunocompetent patients that have not been heavily pretreated, though finding such phase I candidates is often a logistical challenge for the sponsor and principal investigator evaluating new tumor vaccines.

There is general agreement that the optimal patients for such studies are those with minimal residual disease. In addition to immunocompetence, an ideal patient population should not be one where underlying disease is rapidly progressive, as in end-stage or refractory patients (and where traditional chemotherapy models might be more appropriate), but rather in a population that can be expected to remain in study long enough to receive sufficient exposure to develop an immune response. It may therefore be reasonable to conduct studies in patients with early stage of disease or in the appropriate adjuvant setting.

4.1.2. DESIGN

Other ongoing issues in the phase I testing of biological agents such as tumor vaccines is the historical reliance on evaluation of tolerability and dose escalation using the traditional testing paradigm of determination of maximum tolerated dose (MTD). It is clear that with most tumor vaccines, the goal should instead be to identify a pharmacologically/immunologically effective dose or an optimal biological dose on which to base development decisions. An exception to this philosophy may be with viral vector-based tumor vaccine products, which, as indicated earlier, have unique clinical development issues.

Regarding design issues, dose selections that are going to be evaluated in initial studies should cover a broad range to characterize the relationship between dose and immunologic activity. In this way, the goal would be not so much evaluating the differences in toxicity levels, but assessing the differences in the immunologic activity level between dose cohorts through the use of immunological markers as discussed in subheading 3.1. Regarding size of cohorts, instead of the traditional three to six patients per dose-level cohort, consideration should be given to justifying the size of the cohort based upon the number of patients necessary to distinguish immune marker dose effects. Since at this time the appropriate immune-monitoring assays do not exist to characterize the clinical activity of tumor vaccines, clinical trials of many vaccines in development have been unable to address the issue of proper patient cohort sizes to assess dose response.

4.2. Phase II

Important considerations in phase II studies include an exploration of the impact of the dose and dosing regimen on clinical outcome measures. With some tumor vaccines (e.g., dendritic cell vaccines), route of administration appears to be a factor in the degree of immune responsiveness (14,15), potentially altered by the use of immune adjuvants, cytokines, and/or multiple antigens. It has been suggested that these elements could be best explored in a randomized phase II study design that compared various strategies at the same time, rather than performing a series of phase II trials. Simultaneous assessment of the variables in the proper clinical trial setting may remove some confounders and thus allow more meaningful comparisons between groups (16).

As trials with tumor vaccines have generally been conducted in the adjuvant setting or in minimal residual disease states, clinical efficacy end points have focused on survival and progression-free survival. Optimally, phase II trials should have a randomized study design with appropriate internal controls. Unfortunately, many studies have utilized historical controls and it is important to note that these types of designs do not meet the regulatory requirement of “adequate and well-controlled trials” for the licensing approval of a biologic therapy.

4.3. Pivotal Clinical Trials

Issues important in the design of phase II studies are germane in the phase III/pivotal trial setting as well. It is also important to note that, since the body of evidence prior to phase III may not show clear evidence of clinical benefit (as might be seen in cytotoxic chemotherapy trials), then it is likely that more than one randomized, controlled trial may be necessary for initial product licensure. However, once on the market for the approved indication, for the approval of a supplemental indication, it is suggested that thorough discussions occur between the sponsor and CBER to arrive at potentially creative clinical development programs that build on knowledge gained from the initial indication.

Regarding trial end points, survival remains the gold-standard clinical trial endpoint to assess the efficacy of any new cancer regimen, tumor vaccines included, in the minimal residual disease setting. Because of the obvious dilemma that the survival endpoint creates vis à vis large, lengthy studies in potentially small targeted patient populations, it highlights the profoundly important need for the tumor vaccine community to develop valid immune system surrogate markers that indeed correlate with clinical efficacy.

4.4. Surrogate Measures of Clinical Response

The regulatory definition of a surrogate endpoint is “a laboratory measurement or a physical sign used as a substitute for a clinically meaningful endpoint that is a direct measure of how a patient feels, functions or survives, and which is expected to predict the effect of therapy” (57 Federal Register 13234 and 13235, April 1992). The FDA has recognized that durable complete and partial tumor responses are acceptable as surrogate end points in most malignancies. In addition, time-to-progression has been accepted as a surrogate in, for example, the adjuvant setting, and has on occasion been considered an outright measure of efficacy.

It should be noted, however, that effects of tumor vaccines on serologic tumor-associated antigens (e.g., PSA) have not been considered to be a surrogate endpoint for clinical efficacy. The same statement is true for immunological responses against tumor antigens, since in addition to the lack of immune markers for pharmacologic (immunologic) activity, no surrogate marker currently exists related to the clinical efficacy of tumor vaccines.

5. CONCLUSIONS

The last two decades have yielded an explosion of knowledge about the role of the immune system in responding to cancer and some tantalizing data have emerged about the potential clinical value of the complicated class of products known as tumor vaccines. A number of manufacturing, toxicology, clinical pharmacology, and clinical research regulatory issues exist for tumor vaccines that have been outlined in this chapter. The most vexing issues at this time are the lack of a validated surrogate marker for immune-based treatments such as tumor vaccines and the need for better approaches for evaluating the potency and identity of the various tumor vaccine classes. Current efforts seem to reflect our naïveté about the role of the different arms of the immune response in the setting of malignancy and in so doing results to date have failed to meet the requirements for commercial licensure as outlined in federal regulations. Thus despite the enormous promise of these products for patients with cancer, future pathways toward the successful commercial development of tumor vaccines are at the present time at a regulatory crossroads.

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