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Humanized Monoclonals and Other Biological Initiatives

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Introduction

The current phase in the development of new biologicals challenges us to understand how these agents should be used and to find their place in immunosuppressive protocols. In this article, we present a discussion of the biological initiatives in relationship to the T cell response.

Three signals for T cell responses

Resting T cells (G_0) are triggered by antigen presenting cells that present two signals: antigen and costimulation. Antigen (MHC plus peptide) engages TCR and activates T cell tyrosine kinases such as ZAP70, which activate signal transduction pathways such as the calcium-calcineurin pathway and the ras-MAP kinase pathway, the SAPK/JNK pathway, and the NF-κB pathway. These signals lead to the transcription of the genes for cytokines (*e.g.*, IL-2, IL-4, IFN-γ, etc.) and cytokine-like membrane molecules (*e.g.*, CD40 ligand [CD40L] and Fas ligand [FasL]. Thus, the T cell becomes activated (G_1). Certain cytokines, such as IL-2, provide a growth signal for T cells, which permits the T cells to enter cell division. Before dividing, T cells must have an adequate *de novo* synthesis of nucleotides or they will not proceed through the S phase.

A recent development has been the appreciation of the important role of CD40L. CD40L expression is probably triggered mainly by signal 1 (antigen) and activates antigen presenting cells to express their costimulatory activities (*e.g.*, B7). CD40L has an obligatory role in the interaction of T cells with B cells for Ig class switching, and probably with endothelial cells, fibroblasts, and other cells.

An important aspect of lymphocyte responses is that the lymphocytes are programmed to die. In T cells, there are two programs for death: the one that occurs secondary to antigenic stimulation itself and the one that is antigen independent and occurs at the end of the immune response when antigen is withdrawn. The antigen-dependent pathway is triggered by the Fas ligand, and the lymphokine withdrawal pathway occurs via other mechanisms, and is suppressible by the proteins in the Bcl-2 family. We know little about the effects of immunosuppressives on lymphocyte death, and the relationship of lymphocyte death to problems such as T cell anergy and tolerance.

What is immunosuppression?

Immunosuppression is the reduction of undesirable immunologic activity by the destruction or sequestration of immunocompetent cells or by inhibition of an essential, nonredundant, step in the immune response. It is probably preferable to change function or traffic of immune cells than to destroy them. Many tolerance strategies are based on some degree of destruction of immune cells or suppression of immune functions, followed by recovery in the presence of antigen. In general, recovery in the presence of antigen confers at least temporary hyporesponsiveness. This effect probably is inherent in the success of all transplant immunosuppression, which begins with heavy immunosuppression (induction) followed by relatively light immunosuppression after the first 3 to 6 months. There are many components of this host and graft adaptation, such as resolution of injury-induced inflammation in the graft itself.

Identify nonredundant steps in the immune response

The main sources of information come from studies of existing immunosuppressive drugs, from genetic mutations in humans causing immunodeficiency, and from studies of the effects of knockouts. These sources show us the semi-redundant or redundant steps, which when mutated do not produce severe immunodeficiency, but may produce some blunting of the response. For example, IL-2 knock-

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outs suggest that IL-2 is semi-redundant (1), whereas CD40L knockouts display significant immunodeficiency (2–4). In such studies we must distinguish effects on development of lymphocytes from effects on function, which is what we are seeking.

Defects in tyrosine kinases associated with T cell receptor (*e.g.*, ZAP70) (5), defects in CD40 ligand (6,7), in the cytokine receptor γC common chain (8), class II MHC expression (9,10), and defects in purine nucleotide synthesis (11,12) all produce clinically significant immunodeficiencies in the human. In the knockout mice, CD28 defects (13,14), CD40 ligand defects (2–4), and class II transactivator (15) also produce immunodeficiencies. The calcineurin (CN) knockout mouse produces partial depletion of CN and partial immunodeficiency (16).

Immunosuppression can be conceptualized quantitatively as being complete or partial. Complete inhibition means near total suspension of T cell function, which we can achieve with OKT3 with its combination of effect of depleting T cells and blocking their function by saturation of the CD3 mechanism. Partial inhibition can occur by saturating a partially redundant mechanism (*e.g.*, the IL-2 receptor via its specific α chain), or by partially inhibiting a rate-limiting mechanism such as CN, inosine monophosphate dehydrogenase (IMPDH), or target of rapamycin (TOR). In general, oral agents partially inhibit intracellular enzymes: we have shown this with cyclosporine (17) and have some evidence for this with mycophenolate mofetil (18), and we suspect that the same will apply to tacrolimus and sirolimus. Saturation is an easier state to manage clinically than partial saturation, because one can then give an excess of drug and simply "top up" the levels to maintain saturation over time. The new humanized monoclonals against anti-CD25 (IL-2 receptor α chain), daclizumab and basiliximab, act by prolonged saturation of the IL-2 receptor, including both blocking and disrupting (shedding or internalization) of the receptor.

Immunosuppressives can also have qualitative differences. Agents may differ in their ability to promote or prevent tolerance, in their selectivity for certain aspects of the immune system (*e.g.*, activity against B cells) and in their ability to suppress nonspecific inflammation. The control of the inflammation associated with tissue injury is probably important in controlling the immune response against transplants, because cadaver organs are almost uniformly damaged in the brain death and transplantation processes. CTLA4Ig may have effects against injury-induced inflammatory responses (19). Such damage can create inflammation and may account for the higher rejection rates in organs that are injured or stressed, *e.g.*, marginal donors.

Immunosuppression with proteins

Protein-based interventions have the potential for very high specificity, essentially no nonimmune toxicity (nephrotoxicity and other "collateral damage"), saturation, and less need for drug monitoring. The bad news about proteins is their possible antigenicity and the need for parental dosing, and, historically, their high cost. Some proteins produce first dose effects (*e.g.*, OKT3) and trigger host immune responses, which rapidly neutralize the protein and limit its future use. Protein interventions usually only work in extracellular molecules such as membrane receptors and adhesion molecules, or secreted molecules such as cytokines.

Table 1
Some of the Protein or Peptide-Based Biological Immunosuppressives

Antibodies against immune proteins
Polyclonal ALG
Murine monoclonals
Humanized murine monoclonals
Engineered products using natural properties
Solubilized ligands, receptors (IFN-γR)
Ligands or receptor-Ig fusion proteins (CTLA4Ig)
Natural immunosuppressive cytokines
IL-10, IL-4, TGF-β, IFN-γ, IFN-α
Immunotoxins
Natural ligand-ligand (*e.g.*, IL-2-diphtheria toxin) or
Mab-targeted toxins (*e.g.*, anti-CD3—diphtheria toxin conjugate)
Peptides
HLA-based, others

Fusion proteins are made by a natural ligand, by fusing a soluble protein with specificity for a natural structure to the Fc portion of IgG. More common protein interventions are created from antibodies by immunization and selection of antibodies with high specificity. Antibodies may be made in other species and may be polyclonal or monoclonal. The new antibodies are humanized or chimeric monoclonals (*i.e.*, originating as mouse or rat monoclonals) and then having the specificity information in the immunoglobulin genes transferred into human immunoglobulin genes, which are then transfected into mouse hybridoma that secrete the new product with the specificity of the original mouse monoclonal but in a human Ig framework.

There is considerable potential for intravenous immunoglobulin (IVIg) in control of antibody responses and possibly other responses in transplantation (20–23). IVIg may work by triggering inhibitory Fc receptors on immune cells, possibly triggering inhibitory phosphatases such as SHP-1. IVIg has many applications in transplantation but its cost is prohibitive. Perhaps it is revealing a mechanism that can be tapped by cheaper monoclonals.

Table 1 shows a list of some of the protein or peptide-based biological immunosuppressives. Many of these interventions are not being actively developed. For example, cytokines with immunosuppressive potential are all potentially proinflammatory under some circumstances, *i.e.*, double-edged swords. As a result, most current activity in protein

immunosuppression is based on antibodies against immune proteins (polyclonal antilymphocyte globulins made in horses or rabbits, murine monoclonals, and humanized murine monoclonals). The other major initiative is in fusion proteins. Fusion proteins are created by expressing a gene constructed to encode a natural molecule with high affinity for ligand (*e.g.*, CTLA4, which binds to B7 with high affinity) and fusing it with a stable protein which gives it a long half-life (*e.g.*, the hinge and Fc portion of human IgG heavy chain). There are two types of murine monoclonals that have been combined with human IgG: chimeric or fully humanized. Chimeric antibodies have the entire V region of the light and heavy chains transferred on to the human constant regions. This retains all of the specificity of the mouse monoclonal, but with some additional mouse sequences in the V region. The fully humanized antibodies have only the complementarity determining regions from the mouse transferred into the human IgG molecule to replace the human complementarity determining regions. This leaves less "mouseness" but it reduces the affinity for antigen somewhat.

Recent experience with anti-CD25s, basiliximab (chimeric) and daclizumab (humanized), suggests that both strategies reduce antigenicity to very low levels and give monoclonals with half-life equivalent to human IgG (about 22 d). Each dose has effects for several weeks, depending on the dose administered. The monoclonals can be given repeatedly because there is little neutralizing antibody or antidiotype response against them. Even chronic reuse is probably feasible.

Mechanism of action of fusion proteins or the new monoclonals

Fusion proteins or the monoclonals can alter the immune system in many ways. They may delete by lyse immunocytes or change their traffic such that they are taken up in the central lymphoid organs or the reticuloendothelial system. More commonly, they act to either partially disrupt (internalize, shed) a receptor, or to block certain aspects of the receptor so that it becomes nonfunctioning. The studies of the effects of the monoclonals remain unclear. For the moment, we prefer to conceptualize them as blocking the ligand rather than changing the characteristics of the cell bearing the ligand, for the most part. The goal is saturation of the ligand in rendering it nonfunctional for long periods of time. The studies of the anti-CD25s suggest that they act mainly by blocking IL-2Rs, but that they also reduce the number of detectable IL-2Rs, possibly causing disrupting, shedding, or internalization.

CTLA4Ig

CTLA4Ig is capable of blocking B7-1 and B7-2 sites (and possibly other sites), thus suspending costimulation. In theory, suspending costimulation eliminates signal 2 and permits signal 1 to induce tolerance. In practice, it has not yet been shown in higher animals that this will reproducibly be a tolerizing strategy as opposed to simply an immunosuppressive strategy. In rodent models (which are relatively forgiving) this strategy has been shown to work. CTLA4Ig has been used in human psoriasis where it is immunosuppressive. Whether it will go on to human phase I studies in transplantation has not yet been determined. One thing about interfering with B7-CD28 signaling is that it is probably only going to create mild immunosuppression. The CD28 knockout mouse is relatively immunocompetent, indicating that there is a degree of redundancy of the CD28 signal. Nevertheless, it is hoped that the immunosuppression via this signal will create a favorable environment to tolerize the host via the antigens of the graft.

Anti-CD40L

As we have stated, mutations in mouse and human involving CD40L gene create immunodeficiency with hyper IgM (the latter due to the lack of class switching of B cells, which requires T cell signals). It is likely that CD40L plays a role not just in T cell effects but in natural killer effects. Thus, CD40L may have the ability to suppress immune responses, including suppressing IgG producton, suppressing delayed type hypersensitivity responses, and possibly suppressing nonspecific responses.

There has recently been enthusiasm for the use of CTLA4Ig along with anti-CD40L (24,25). This combination is powerful, and combined with a graft produces a relatively long lasting state of hyporesponsiveness against the graft. However, there is still no evidence that this state will survive long-term without decaying. Recent evidence suggests that some of the animals treated in this way may get late deterioration of the graft and may not be stable in the absence of immunosuppression as it had originally been hoped.

Humanized monoclonals against the IL-2 receptor

In articles that are currently in preparation or in press, four large clinical trials have explored the potential of a fully humanized monoclonal (HAT, zenapax, daclizumab) or a chimeric monoclonal (simulect, CHI-621, basiliximab) for their effect on the IL-2 receptor (specifically the α chain or CD25). Because of a degree of redundancy of this receptor, saturation of the receptor does not produce severe immunosuppression. Thus, the four studies of the two monoclonals all produce relatively similar results, as presented in abstract form: about one-third reduction in acute rejection, with essentially no side effects. In particular, the studies show no evidence of increased infections, even CMV. This is the first time that an immunosuppressive effect has been

TABLE 2
Other Monoclonal and Fusion Protein Targets

T cell, receptor, coreceptor, and triggering
TCR
CD3
CD4, 8
CD45 isoforms
T cell costimulation
CD28, B7, CTLA4Ig
T cell effector molecule
CD40L, FasL
Other T cell molecules
Anti-CD2
Anti-CD7
Cytokine receptors
CD25, chemokine Rs
Adhesion molecules
CD54
CD11a or CD18

obtained without collateral damage in other organs in infectors. Although the immunosuppression is obviously relatively mild, they seem to be very stable and well tolerated. The potential of this intervention is just beginning to be explored. It must be stated, however, that the degree of suppression is maintained against a background of full immunosuppression with cyclosporine plus or minus azathioprine. How immunosuppressive these monoclonals will be without such a background will remain to be seen.

The remarkable aspects about the recent experience with chimeric and humanized antibodies is the absence of side effects, except for the potential to have excessive immunosuppression, and the lack of late deterioration of the effect (on maintenance CsA). This is a remarkable development: immunosuppressive drugs with no nonimmune toxicity have previously been considered impossible to develop (26,27).

Other monoclonals

Table 2 shows a list of other monoclonals. The potential of these interventions is enormous. They could be used for reversal of rejection, stabilizing patients with refractory rejection, and induction to prevent rejection. They also have potential as maintenance therapies long-term, in the primary prevention of rejection. There is no essential reason why a well-tolerated monoclonal that saturates a semiredundant target could not be used long-term. The potential of these interventions to produce a state of tolerance when combined with graft antigens or with bone marrow is much discussed, but is currently unknown. To date, no protein interventions when combined with antigen produced indefinite graft survival without some late rejection and graft loss.

The use of protein interventions to suppress the inflammation associated with graft injury from nonspecific factors in an increasing issue. Recently, Sayegh and collaborators (19) have shown that CTLA4Ig can abrogate the nonspecific inflammatory response to graft injury. The suppression of the injury response may be one of the major new ways of using protein interventions. Suspending nonspecific inflammation may reduce costimulation and MHC expression, and, thus, create an environment favorable to tolerance.

Tolerance

Tolerance is defined operationally in transplantation as long-term graft survival without immunosuppression. Tolerance can be due to clonal deletion, anergy, regulation, deviation, or ignorance. Some degree of partial tolerance is probably the basis of success of all transplantation. Tolerance can be central or peripheral. Central means that the tolerance occurs in the thymus, and peripheral means that it is maintained by mechanisms outside of the thymus operating on circulating lymphocytes. Central tolerance has a tendency to be robust, but to require a high degree of engraftment of bone marrow-derived cells in the thymus (not microchimerism but mixed allogeneic chimerism) (28,29). Some peripheral tolerance probably always exists in successful transplants, but peripheral tolerance may be inherently somewhat unstable. It tends to be dynamic, flexible, and potentially reversible, rather than a reliable and robust long-term state.

There is a belief that stable long-term tolerance can be induced by short courses of immunosuppressive agents, with or without injections of donor bone marrow. The recent impressive early results with agents such as anti-CD40L plus CTLA4Ig (24,25) and immunotoxin (30) cannot be interpreted as proof of long-term tolerance. Such forms of immunosuppression last many months and the grafts will probably reject over time (*e.g.*, 2–3 years). The belief is that this form of tolerance will then survive indefinitely through years or for the life of the host. Despite the considerable energy being devoted to achieving this goal, there is little current evidence that this is realistic for any immunosuppressive strategy in a complex human population. One of the problems with tolerance is how one would evaluate it: leaving people off immunosuppressive drugs and waiting for them to reject is probably not acceptable. It would be desirable to have some laboratory measurement to evaluate tolerance. Nevertheless, tolerance will be high on the agenda of our goals in the next few years. Some would say that tolerance exists when persons have stopped their immunosuppressives and have not yet rejected. This seems to us to be like saying that a person who jumps off a building is flying until they hit the ground. Some measurement of immunologic stability would be highly desirable before we put people at risk for rejection and graft loss.

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