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Diminished neo-antigen response to keyhole limpet hemocyanin (KLH) vaccines in patients after treatment with chemotherapy or hematopoietic cell transplantation

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Abstract

Relapse is the most common cause of treatment failure for advanced cancer, even those treated with autologous hematopoietic cell transplantation (HCT). Effective tumor-specific immunotherapy may decrease relapse, however, this will fail if the immune system is unable to respond. We developed a strategy to test immune responses with a single injection of the bona fide neo-antigen KLH. The model was first tested in 37 normal volunteers using three KLH vaccines: Intracel KLH, Biosyn KLH, and Biosyn KLH + adjuvant. Despite finding the immunogenic epitope conserved in both products, intact Intracel KLH induced a better response compared to a purified 350/390 kDA subunit of KLH contained in the Biosyn KLH product. Addition of a synthetic oil adjuvant (Montanide ISA51) restored the response to a single injection of Biosyn KLH. A quantitative readout measured by a KLH-specific cellular and humoral response with isotype switching 1 month after KLH vaccination was established. To test the integrity of the adaptive immune response in cancer patients, we vaccinated 14 patients post-HCT and 19 patients with advanced cancer with KLH vaccines that elicited a 100% response rate in normal volunteers. In marked contrast to normal subjects, both responses were significantly impaired up to 16 months after autologous HCT with an intermediate response in advanced cancer patients. KLH vaccines are safe and require only a single injection to test neo-antigen responses providing an optimal platform for definitive testing of strategies to improve diminished immune recovery after chemotherapy or post-HCT.

Keywords: Immune response; Vaccines; Neo-antigen; Cancer; Hematopoietic cell transplantation; Humoral response; Cellular response; Immune defect

Introduction

Decreases in lymphocyte numbers, cytokines, and immunoglobulin levels have been described in patients with cancer after autologous and allogeneic HCT [1–8]. The functional impact of these changes on an antigen-specific immune response is less well known due to a paucity of vaccines with definitive readouts. Vaccines may be neo-antigens in some patients due to lack of prior exposure or by design [9–12], but sensitive and quantitative measurements of both cellular and humoral responses are not available for monitoring vaccination responses. KLH was chosen for these studies because it is immunogenic in virtually 100% of vertebrate animals [13,14]. The goal of this study is to develop a vaccine strategy with a single injection of a neo-antigen to assess the immune integrity of cancer patients.

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Materials and methods

Subject cohorts and blood samples

Normal adult volunteers (n = 37) and cancer patients were stratified into two groups: (1) those with metastatic melanoma (n = 11) or renal cell carcinoma (n = 8) or (2) recovered from autologous HCT for CML (n = 1), lymphoma (n = 2), myeloma (n = 8), or breast cancer (n = 3). Blood was drawn before and 1 month after immunization to measure KLH-specific cellular immune responses. Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll–Hypaque (Amersham Biosciences, Uppsala, Sweden) separation. Where indicated, PBMCs were enriched for $CD4^+$ or $CD8^+$ cells by CD8 or CD4 depletion, respectively, using MACS columns (Miltenyi, Auburn, CA). Written informed consent was obtained from all subjects with approval from the University of Minnesota Institutional Review Board.

Vaccine products

KLH (Intracel, Rockville, MD or Biosyn Corp, Carlsbad, CA) was given as a single 1 mg subcutaneous injection under INDs sponsored by J. Miller (BB-IND 6745, BB-IND 10430). These products differ in purification strategies. Intracel KLH is isolated using a process that preserves the high molecular weight structure while minimizing low molecular weight degradation products (www.intracel.com). Purity is greater than 95% by HPLC. Biosyn KLH is a purified and standardized immunocyanin containing KLH subunits (350/390 kDA) in a lyophilized powder (www.biosyncorp.com). Each vial contains a protein concentration of 1 mg with glycine, sodium hydroxide, sodium chloride, and sucrose, accounting for a total powder weight of 54.6 mg. Each 1 mg vial was reconstituted in 1 ml sterile water for injection. Pharmacy grade Tetanus Toxoid Adsorbed, PUROGENATED® (TT, Lederle), is a sterile preparation of refined tetanus toxoid. Each 0.5 ml dose is formulated to contain 5 Lf units of tetanus toxoid and 0.11 mg of aluminum from the aluminum phosphate adjuvant for intramuscular use only. The residual free formaldehyde content by assay is <0.02. The tetanus toxoid induces at least 2 neutralizing units/ml of serum in the guinea pig potency test.

Where indicated, water soluble Biosyn KLH was emulsified with Montanide ISA-51 (Seppic, Inc., Fairfield, NJ, www.seppic.com). Montanide ISA-51, NSC 675756, is an oil-based adjuvant product similar to incomplete Freund's adjuvant which, when mixed with a water-based solution on 1:1 w/w ratio, forms a water-in-oil emulsion. It consists of a purified oil, Drakol VR, and a surfactant, mannide oleate. The KLH 1 mg vial was first reconstituted in 0.5 ml sterile water. Once solubilized, 0.6 ml of Montanide ISA was added to the vial and placed in a vortex machine at highest speed for 12 min. Because neither the KLH solution nor the Montanide ISA-51 contains preservatives or bacteriostatics, the vaccine was administered as soon as possible after preparation (4 h or less).

Proliferation assays

Proliferation assays were performed in 96-well round-bottom plate using cells (100,000 cells/well) without or with 2 μg/well KLH (Calbiochem–Novabiochem Corporation, San Diego, CA) or 0.2 μg/well tetanus toxoid (TT) (Wyeth–Ayerst Pharmaceuticals, Radnor, CA). The plate was incubated for 7 days at 37°C, 5% CO_2. For the final 16 h of incubation, the cells were pulsed with 1 μCi/well of tritium labeled thymidine (MP Biomedicals, Irvine, CA) then harvested onto filters using a plate harvester (Perkin-Elmer, Boston, MA). Filters were counted on a Matrix 9600 (Perkin-Elmer). Responder frequencies were calculated using the number of positive wells, which were greater than 5 standard deviations above the negative wells without antigen.

Enzyme-linked immunospot (ELISPOT) assay for cellular responses to KLH and TT

After showing similar results with fresh and frozen cells, cryopreserved PBMCs were thawed and assayed from blood collected before and after vaccination. Thawed cells were left overnight at 37°C then counted and plated at 2.5×10^5/well in Millipore Multiscreen 96-well nitrocellulose plates (Millipore, Bedford, MN) that had been coated with anti-IFNγ antibody (BD PharMingen, San Diego, CA). Cells (2.5×10^5 cells/well for 3–6 replicates) were incubated at 37°C for 20 h without antigen (negative control) or with antigen (tetanus toxoid at 2 μg/ml or KLH at 10 μg/ml) and washed thoroughly before adding a second biotin-labeled anti-IFNγ antibody (BD PharMingen). After incubation for 2.5 h at room temperature, plates were washed and IFNγ-positive spots detected by incubating with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch, Westgrover, PA) followed by addition of 5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitro Blue Tetrazolium (Sigma, St. Louis, MO) as substrate. Plates are washed with distilled water to end the reaction, and spots are counted using the Carl Zeiss Vision ELISPOT reader (Zeis, Thornwood, NY). Samples that did not show a response to a positive control for sample integrity (PWM, Sigma) with >100 spots per million cells were excluded from further analysis (n = 2).

Antigen-specific immunoglobulin (Ig) responses and flow cytometry

Isotype-specific Igs and KLH-specific Igs were measured by sandwich ELISA. ELISA plates (Costar) were coated with KLH (Intracel or Biosyn) at 20 μg/ml overnight at RT, blocked with 1% BSA/5% sucrose in PBS, loaded with pre- and post-vaccine sera (dilutions of 1:50, 1:100, 1:200, 1:400, and 1:800), and incubated for 2 h at 37°C. Bound Igs

were detected with peroxidase-labeled isotype-specific sheep–anti-human reagents and tetramethylbenzidine substrate. Plates were read at 450 nm (with 540 nm correction). Optical densities were compared to standard curves generated with purified human Ig isotypes bound to wells, and quantitative measures of anti-KLH Igs (in μg/ml) were interpolated. Determinations of total IgG1, IgG2, and TT-specific IgG levels were made using commercially available kits. Total IgM levels were done by ELISA on plates coated with sheep–anti-human μchain Ab. Samples were serially diluted 1:25,000, 1:50,000, and 1:100,000, incubated for 2 h at room temperature, and detected with peroxidase-labeled sheep–anti-IgMμchain. Quantitative measurement of IgM levels was interpolated from standard curves. All antibodies, wash buffer, substrate, and commercial kits were purchased from The Binding Site (San Diego, CA).

Immunophenotypic analysis of PBMCs was performed using 3-color analysis on a FACSCalibur (Becton Dickenson, San Jose, CA) with CELLQuest Pro software (Becton Dickenson). Cells were stained with the following monoclonal antibodies (mAbs): fluorescein isothiocyanate (FITC)-conjugated CD4, Phycoerythrin (PE)-conjugated CD8, peridinin chlorophyll A protein (PerCP)-conjugated SK7 (anti-CD3), and isotype controls (all from Becton Dickenson).

Statistics

Paired *t* tests were conducted on the $\log_{10}$-transformed data to determine whether KLH induced an IgG1, IgG2, or IgM response in normal volunteers. A linear model was used to test the effect of the KLH product coated in the ELISA assay on the change in immunoglobulin levels (pre-immune–post-vaccine). Cutoff points for identification of responders to the vaccines were determined using the method of Le et al. [15], using the baseline response for the normal subjects. Patients with melanoma or renal cancer were combined and analyzed as the cancer cohort. General linear models were used to determine the effects of patient group, vaccine status (pre-immune vs. post-vaccine), and the interaction between patient group and vaccine status on the $\log_{10}$-transformed KLH and TT ELISPOT data. Partial correlation coefficients were obtained between absolute CD4 counts and the KLH-specific Ig responses to examine the association between the absolute CD4 counts and KLH-

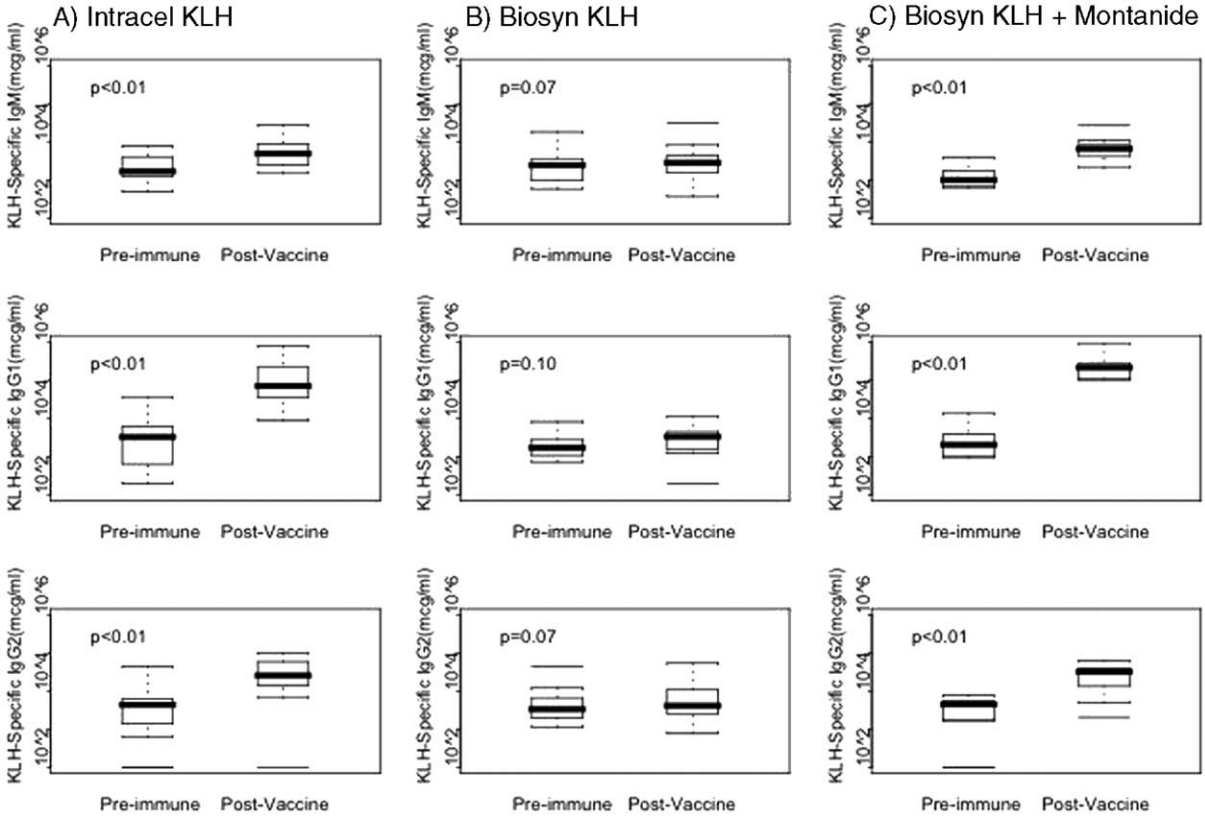

Fig. 1. Normal volunteers exhibit a humoral response and isotype switching to neo-antigen KLH vaccines. Normal subjects received 1 mg Intracel KLH (n = 17), Biosyn KLH (n = 10), or the adjuvant Montanide ISA-51 + Biosyn KLH (n = 10). The humoral response was determined by ELISA 28 days after vaccination. Boxplots are shown for from baseline pre-vaccine and 28 days later for KLH-IgM, KLH-IgG1, and KLH-IgG2. All normal donors responded to Intracel KLH alone and Biosyn KLH administered with the Montanide ISA-51 adjuvant. In contrast, measurable responses to Biosyn KLH alone as a single vaccine administration without adjuvant were minimal.

specific Ig responses after removing the unwanted effects of the KLH vaccine product [16]. The overall level of significance for each test was set to 0.05.

Results

KLH was first used in normal subjects to establish quantitative immune readouts. Intracel KLH vaccination (containing the intact KLH molecule) induced a potent IgM, IgG1, and IgG2 response in normal volunteers within 1 month of administration (n = 17) (Fig. 1). However, because of sporadic availability of Intracel KLH, Biosyn KLH, produced as 350/390 kDA subunits of KLH, was then tested. In marked contrast to Intracel KLH, Biosyn KLH alone did not significantly induce either a humoral (Fig. 1) or cellular response (data not shown). The finding that serum from normal subjects vaccinated with intracel KLH bound to both Calbiochem and Biosyn KLH-coated plates in the ELISA assay (Fig. 2) suggested that the lack of response was not due to a loss of important epitopes between products. We hypothesized that lack of a response to a single Biosyn KLH immunization was due to an adjuvant property contained in the intact Intracel KLH product. Therefore, Biosyn KLH was emulsified with a synthetic incomplete Freund's adjuvant (IFA, Montanide ISA-51), which completely restored immunogenicity to a single 1 mg KLH test vaccine in normal subjects (Fig. 1). For the Biosyn KLH product plus Montanide ISA-51 adjuvant, 100% of normal volunteers developed a humoral (ELISA) response. Because the amplitude of the humoral response to Intracel KLH was lower than the response to Biosyn KLH plus Montanide ISA-51 and Intracel KLH became unavailable, results were combined for presentation with response definitions based on the Biosyn vaccine.

The normal brisk response to a single KLH vaccine was compared to that seen with patients after autologous HCT and with patients who have metastatic melanoma or renal cell carcinoma (Figs. 3A–C, Table 1). A positive response was determined according to the methods of Le et al. [15]. Patients after autologous HCT exhibited a marked defect in KLH-specific IgM, IgG1 (Th2-dependent), and IgG2 (Th1-dependent) responses with little evidence for isotype switching. Lower IgG2 levels might be expected since HCT patients have decreased total IgG2 levels after trans-

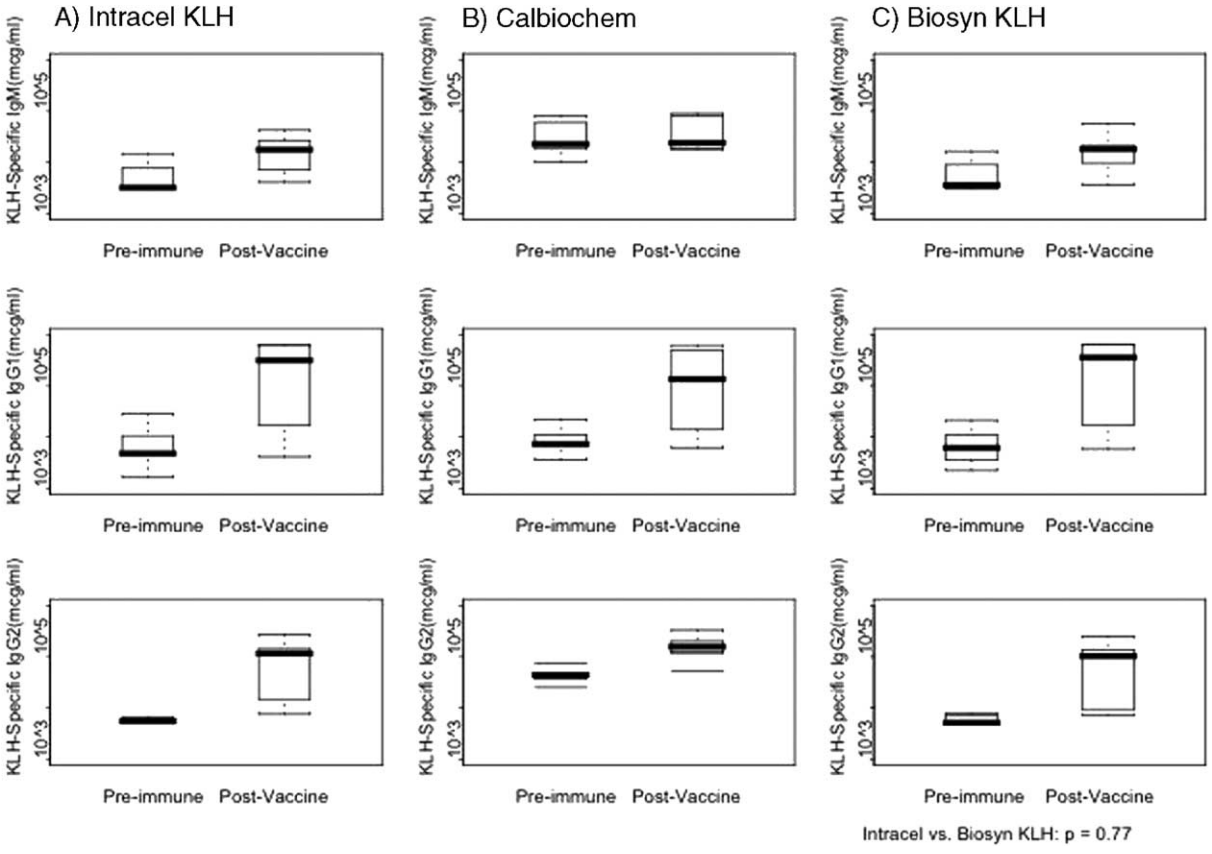

Fig. 2. All KLH products contain the immunogenic epitope recognized by isotype-specific immunoglobulins induced in vivo. Serum was evaluated from normal subjects before (pre-immune) and 28 days after a single 1 mg. Intracel KLH vaccine. An isotype-specific KLH sandwich ELISA assay (Materials and methods) was designed to recognize KLH tested from Intracel, Calbiochem, and Biosyn. Boxplots are shown for from baseline pre-vaccine and 28 days later for KLH-IgM, KLH-IgG1, and KLH-IgG2. There was no difference between the measurement of isotype-specific immunoblobulins in the ELISA assay and KLH from multiple sources, suggesting that the same immunogenic epitope was contained in all KLH products tested.

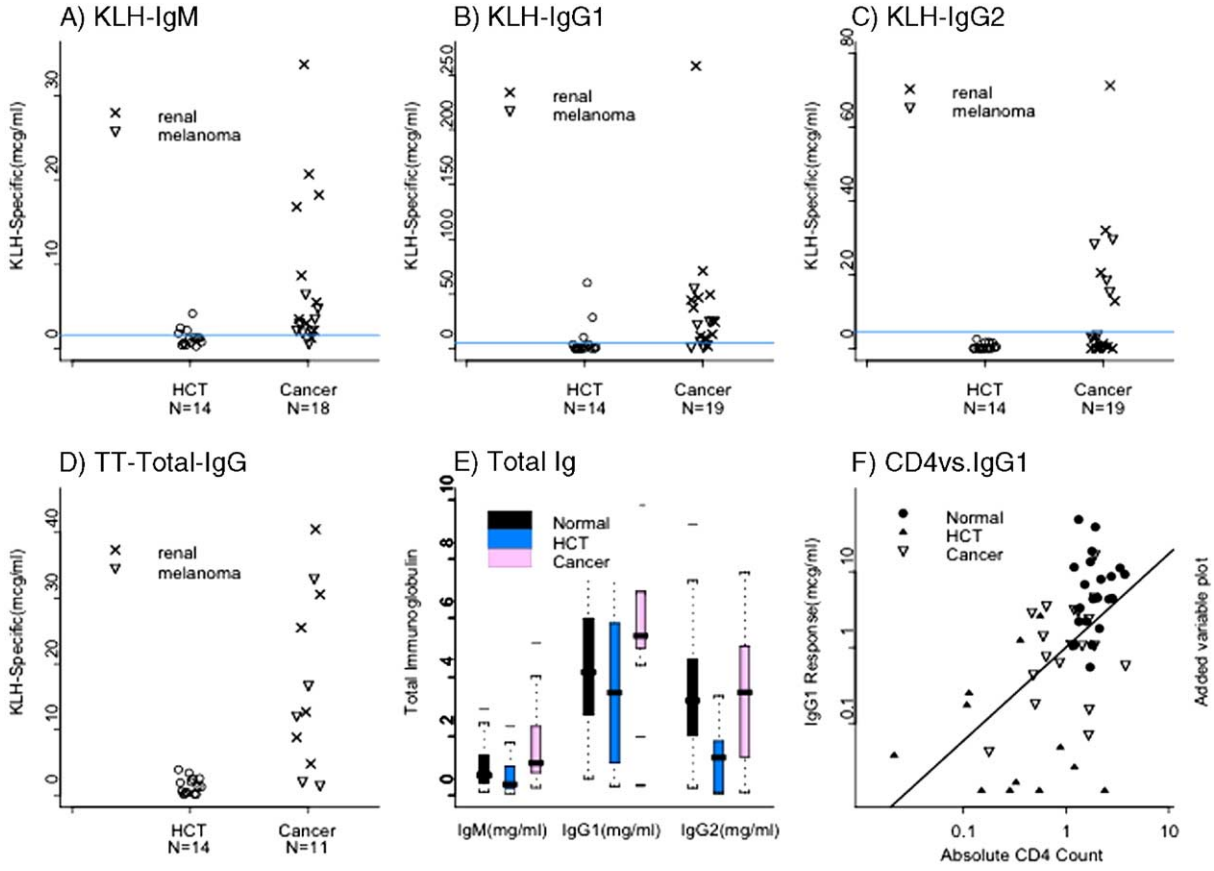

Fig. 3. Patients after HCT or with metastatic cancer exhibit a diminished humoral response to KLH vaccines. Patients after autologous HCT (HCT) or patients with metastatic renal cell carcinoma or melanoma (Cancer) received a single 1 mg. injection of KLH (either Intracel KLH or Biosyn KLH + Montanide ISA-51). The humoral response was determined 28 days after vaccination as indicated. Individual responses are shown for (A) KLH-IgM, (B) KLH-IgG1, (C) KLH-IgG2, and (D) TT-Total IgG. A positive response is indicated for KLH with a horizontal line for IgG1 (5.5 mcg/ml), IgG2 (4.7 mcg/ml), and IgM (1.6 mcg/ml), using the baseline response for the normal subjects receiving Biosyn KLH + Montanide ISA-51 adjuvant. (E) Boxplots of total IgM, IgG1, and IgG2 immunoglobulin are shown for each cohort, including normal volunteers. (F) An added variable plot representing the partial correlation, taking effects of the KLH product into account, between the absolute CD4 count and the KLH-IgG1 response for all subjects (patients and normals).

plant as compared to normal volunteers (Fig. 3E). In contrast, total IgG1 and IgM Ig levels were not significantly different between normal subjects and HCT patients, suggesting a diminished ability of HCT patients to mount a specific IgM and IgG1 response to neo-antigen. Patients with metastatic cancer exhibited a variable response, while

Table 1
Humoral response to KLH in normal volunteers, cancer patients, and patients after HCT

	Intracel			Biosyn + Montanide		
	Pre-mean (SD)	Post-mean (SD)	*P* value	Pre-mean (SD)	Post-mean (SD)	*P* value
Normal subjects						
IgG1	5508.5 (8404.3)	207,175.0 (271,965.2)	<0.0001	4157.8 (4686.7)	280,368.5 (244,756.2)	<0.0001
IgG2	6403.6 (10,229.6)	38,655.5 (30,036.0)	<0.0001	3814.5 (2506.5)	28,377.3 (19,176.2)	<0.0001
IgM	2559.6 (1978.4)	7359.1 (7046.2)	<0.0001	1381.0 (990.4)	8159.9 (7114.5)	<0.0001
Cancer patients						
IgG1	1699.5 (1538.8)	47,458.8 (67,314.9)	<0.0001	1334.7 (995.9)	15,396.8 (11,152.0)	0.0091
IgG2	3323.8 (2433.7)	18,080.8 (19,834.9)	0.0004	301.8 (312.2)	1016.5 (1340.8)	0.1019
IgM	2219.2 (1752.3)	8599.8 (10,081.0)	0.0016	2286.6 (1738.3)	5764.6 (6367.2)	0.1410
Patients after autologous HCT						
IgG1	870.4 (1181.7)	2195.6 (3910.3)	0.8354	745.0 (395.2)	13,666.9 (22,870.7)	0.0992
IgG2	642.3 (943.4)	910.4 (1061.1)	0.3328	432.0 (878.4)	426.0 (616.8)	0.2535
IgM	1225.0 (682.6)	1316.3 (852.1)	0.8648	653.0 (144.6)	1405.0 (1281.6)	0.1312

All immunoglobulin responses are in mcg/ml.

some had an apparent normal response to KLH, and some subjects mounted a minimal response. This finding is potentially significant as these subjects were co-enrolled on a trial where therapeutic vaccines were administered. The TT IgG-specific response was also severely diminished after HCT (Fig. 3D).

Since $CD4^+$ T-cell responses are needed for Ig isotype switching, a limiting dilution proliferation assay was performed using fresh cells cultured in the absence or presence of KLH. Although normal subjects increased the frequency of KLH-responsive cells (from a frequency of <1:200,000 to 1:6191–30,558) obtained after KLH vaccine ($n = 5$), only 1 of 7 HCT subjects showed a similar response in a breast cancer patient immunized 16 months after HCT (data not shown). This subject showed no humoral response. Because the background was sometimes high in proliferation assays with normal volunteers, the ELISPOT assay was used to detect IFNγ-producing cells as a more consistent way to quantitate KLH-specific responses. CD4- and CD8-depletion experiments showed that 75–100% of KLH responding T cells and 77–100% of TT responding T cells in this assay were $CD4^+$. Marked defects in cellular responses were seen in patients who received HCT (Fig. 4). In our study, this diminished cellular response in cancer and after HCT corresponded to a decrease in absolute CD4 counts between the normal (891 ± 267/mm^3), solid tumor (571 ± 348/mm^3), and HCT (259 ± 319/mm^3) cohorts (P = <0.004 for HCT and solid tumor patients compared to normal, Kruskal–Wallis test). Including all subjects, absolute CD4 counts also correlated with the KLH-specific IgG1 response ($r = 0.559$, P = <0.001, Fig. 3F) and with IgG2 ($r = 0.467$, P = <0.001) and IgM ($r = 0.396$, $P = 0.005$).

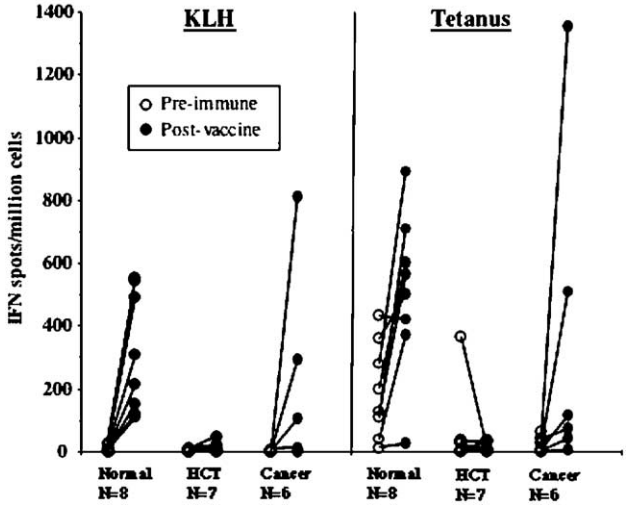

Fig. 4. Patients after HCT exhibit a marked defect in cellular responses to neo-antigen KLH vaccines. The pre-immune and post-vaccine ELISPOT response is shown for KLH and tetanus.

Discussion

We have established a neo-antigen vaccine strategy. KLH vaccines are safe, readouts are quantitative, and 100% of normal volunteers require only a single injection to test cellular and humoral neo-antigen responses, including Ig isotype switching with quantitative cellular and humoral readouts. Applying this strategy to patients with cancer and especially those patients after autologous HCT, neo-antigen responses are significantly diminished.

It is generally accepted that patients with advanced cancer have depressed immune responses. Defects in responses have been demonstrated both for tumor antigens [17–19] as well as a more global defect in immune responses [20–22]. There are multiple mechanisms accounting for the observed immune defects in cancer patients. In one study, dendritic cells (DCs) from patients with advanced breast cancer failed to stimulate allogeneic responses in normal T cells, while the patient T cells responded normally to control allogeneic DCs as well as to immobilized anti-CD3 antibody, suggesting that the immune defect lies at the level of the antigen presenting cell [23]. Another study indicated a systemic defect in maturation of DCs as a result of overproduction of VEGF, a factor which also promotes tumor angiogenesis [24]. VEGF itself has also been suggested to directly interfere with T-cell differentiation [25,26]. Associated with a decrease in mature DCs in cancer patients is an increase in immature myeloid cells which have been found to suppress antigen-specific T-cell responses, possibly through the production of reactive oxygen species, particularly hydrogen peroxide [27–29]. An immature phenotype might be related to the observation that DCs from cancer patients express low levels of costimulatory molecules, which could result in failure to effectively present antigens [20,30]. Another study revealed that a subset of human DCs that express the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) induce tolerance rather than immunity. Such IDO^+ DCs are found in large numbers in some tumor draining lymph nodes and may contribute to immunologic unresponsiveness to tumor antigens [31]. In addition to defects in antigen presentation, recent studies have also revealed the presence of high numbers of immune suppressive $CD4^+25^+$ T regulatory cells in persons with various types of advanced cancer. These regulatory cells have been shown to block both CD4 and CD8 T-cell responses [32–37]. Although the current study does not allow discrimination between these mechanisms, KLH does allow us to test whether a response is intact and to what degree.

Functional immune recovery can be delayed for long periods of time after autologous or allogeneic HCT. For example, Yamagami et al. observed that T cells obtained after HCT had a profound defect in intracellular calcium mobilization after TCR engagement by anti-CD3 mAb [38]. The magnitude of delayed immune recovery is exaggerated after allogeneic transplant by associated pharmacologic

immunosuppression, donor:recipient HLA histocompatibility, and graft versus host disease [6,39–41]. In the autologous setting, diminished immunity is variable as observed clinically by less infectious complications. Our diminished KLH response after autologous HCT differs from that seen by others. Several investigators have clearly demonstrated the feasibility of using KLH conjugated with idiotype for B-cell malignancies [14,42,43]. In a report by Reichardt et al. in myeloma patients receiving idiotype-conjugated KLH pulsed dendritic cells, 11 of 12 myeloma patients were able to produce a KLH-specific proliferative response, but Ig isotype switching was not tested [42,43]. The difference between these studies (exogenous KLH pulsed DCs) and ours (KLH presented through endogenous DCs) supports a defect in antigen presentation. However, the defect is still likely multifactorial given the low CD4 counts as a manifestation of deficient thymopoiesis as described after high-dose chemotherapy and autologous transplantation [44,45].

In summary, neo-antigen KLH vaccines allow quantitative readouts for assessing immune integrity in cancer patients and after HCT. These results have important implications for tumor vaccine strategies in these settings. KLH vaccines will serve as an optimal platform for definitive testing of strategies to promote immune reconstitution or novel immune adjuvants.

Acknowledgments

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