Generation of Immunity to the HER-2/*neu* Oncogenic Protein in Patients with Breast and Ovarian Cancer Using a Peptide-based Vaccine[1]

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ABSTRACT

HER-2/*neu* is a "self" tumor antigen that is overexpressed in 15–30% of human adenocarinomas. Vaccine strategies directed against HER-2/*neu* and other self tumor antigens require development of methods to overcome immune tolerance to self-proteins. In rats, rat *neu* peptide vaccines have been shown to be an effective way of circumventing tolerance to rat *neu* protein and generating rat *neu*-specific immunity. The present report validates that a similar peptide-based vaccine formulation is effective for inducing T-cell immunity to HER-2/*neu* protein in humans with breast and ovarian cancer. The vaccine formulation included groups of peptides derived from the HER-2/*neu* extracellular domain (ECD) or intracellular domain (ICD) mixed with granulocyte macrophage colony stimulating factor as an adjuvant. These peptides were 15–18 amino acids in length and designed to elicit a CD4 T helper-specific immune response. Patients underwent intradermal immunization once a month for a total of two to six immunizations. To date, all of the patients immunized with HER-2/*neu* peptides developed HER-2/*neu* peptide-specific T-cell responses. The majority of patients (six of eight) also developed HER-2/*neu* protein-specific responses. Responses to HER-2/*neu* protein occurred with epitope spreading. Immune T cells elicited by vaccination were shown to migrate outside the peripheral circulation by virtue of generating delayed type hypersensitivity responses distant from the vaccine site, which indicated the potential ability to traffic to the site of tumor. The use of peptide-based vaccines may be a simple, yet effective, vaccine strategy for immunizing humans to oncogenic self-proteins.

INTRODUCTION

HER-2/*neu* is an oncogene that is activated by gene amplification with the increased expression of a normal gene product. As an overexpressed normal protein, HER-2/*neu* protein is an example of the recent "paradigm shift" in tumor immunology, which suggests that self-proteins can serve as tumor antigens (1). The most notable examples are in melanoma in which major proteins implicated in the tumor-specific immune response are nonmutated antigens expressed by some normal tissues, *e.g.,* MAGE and gp100. Thus, a current issue for the development of cancer vaccines is how best to induce T-cell immunity to "self" tumor antigens.

Cancer vaccines targeting self tumor antigens must overcome immunological tolerance. Peptide vaccines may offer an advantage over more classic methods of tumor vaccination that rely on using intact protein or tumor cells as immunogens. Tolerance may be directed toward immunodominant epitopes of self-proteins, which are toleragenic (2). In animal model systems, tolerance to self-proteins can be circumvented by targeting the immune response to nonimmunodominant peptide portions of the self-tumor antigen, *i.e.,* a subdominant epitope. However, there are no standard regimens for immunizing humans to peptide portions of self-tumor antigens.

Initial studies to develop a peptide based HER-2/*neu* vaccine were performed in a rat model (3). Rat *neu* protein is 89% homologous to human HER-2/*neu* protein. No T-cell or antibody responses were observed in animals immunized with intact rat *neu* protein, which indicated that rats are tolerant to whole rat *neu* protein. In similar experiments, others have shown that rats are tolerant to immunization with rat *neu* protein expressed by recombinant vaccinia virus (4). By marked contrast, tolerance to rat *neu* protein in rats could be circumvented by immunization with a peptide-based vaccine (3). Rats were immunized with rat *neu* peptides designed for eliciting CD4+ T-cell responses. T-cell and antibody responses specific for both the immunizing peptides and protein were generated.

There is no standard adjuvant for immunizing humans to peptides, especially self-peptides. Studies in rats established that GM-CSF[3] is a potent adjuvant for the generation of immune responses to rat *neu* peptides (5). Rat *neu* peptides inoculated

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[3] The abbreviations used are: GM-CSF, granulocyte macrophage colony stimulating factor; DC, dendritic cell; DTH, delayed type hypersensitivity; ECD, extracellular domain of the HER-2/*neu* protein; ICD, intracellular domain of the HER-2/*neu* protein; PBMC, peripheral blood mononuclear cell; SI, stimulation index; HLA, human lymphocyte antigen; i.d., intradermal/intradermally; APC, antigen-presenting cell.

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with GM-CSF could elicit a strong DTH response, whereas peptides alone were nonimmunogenic (5). The peptide-based vaccine using GM-CSF was most effective in rats when injected i.d. *versus* s.c., presumably because of the role of i.d. GM-CSF as a chemoattractant and growth and differentiation factor for dermal DCs (6). Thus, the present study tested a HER-2/*neu* peptide vaccine injected i.d. with GM-CSF.

Our preliminary clinical studies have focused on eliciting T-helper responses using longer peptides, 15–18 amino acids in length. Our initial vaccination strategies have concentrated on the HER-2/*neu*-specific CD4+ T-helper response for several reasons. The preexistent HER-2/*neu*-specific immune responses detected in patients is, for the most part, low level (7). A vigorous T-helper response may serve to augment the production of HER-2/*neu* antibodies and/or HER-2/*neu*-specific cytotoxic T cells, both of which could potentially mediate an antitumor effect. The importance of the CD4+ helper T cells in mediating an antitumor response is increasingly being emphasized. As an example, a mouse leukemia model, FBL-3, has been instructive in determining how to generate T cells that will eradicate tumor and in defining the role of T cells in tumor eradication (8). Experiments have shown that for CD8+ cells to be curative, it is necessary to concurrently inject CD4+ cells or administer exogenous interleukin 2 (9). Finally, CD4+ T cells play a major role in the maintenance of immunological memory. The generation of a lasting immune response requires T help. Our initial clinical studies would attempt to generate a higher magnitude HER-2/*neu* T-helper immune response.

In this interim summary, we describe results from the first eight patients, vaccinated with groups of peptides derived from the natural sequence of either the HER-2/*neu* ECD or the HER-2/*neu* ICD. Peptide-specific T-cell responses were elicited in all eight of the patients. In most patients, the elicited T cells responded to HER-2/*neu* protein as well as to peptides. Interestingly, epitope spreading (generation of an immune response to portions of the protein not included in the vaccine) was observed. The purpose of this initial report is to demonstrate the use of peptide-based vaccines using GM-CSF as an adjuvant, which seems to be an effective method for immunizing patients to HER-2/*neu* protein.

PATIENTS AND METHODS

Subjects. The University of Washington Human Subjects Division and the United States Food and Drug Administration approved the study. The purpose of this Phase I study was to evaluate the immunogenicity of HER-2/*neu* peptide-based vaccines—as well as the safety of use—in patients with HER-2/*neu*-overexpressing tumors. Patients with stage III or IV breast and ovarian cancer were eligible for the study if the following criteria were met: (*a*) HER-2/*neu* protein overexpression in the primary tumor or metastasis; (*b*) prior treatment, having either no detectable cancer or minimal residual disease stable on hormonal- or radiotherapy; (*c*) a competent immune system, as measured by responsiveness to a minimum of 2 of 7 recall antigens by skin testing with CMI Multitest (Connaught Labs, Swiftwater, PA); (*d*) the clinical expectation to remain off any immunosuppressive therapy for 6 months; and (*e*) signed informed consent. Patients were randomized to receive a vaccine of peptides derived from the ECD or the ICD of the HER-2/*neu* protein. Subjects were to be immunized once a month for 6 months. At the end of six immunizations, patients were skin-tested against their immunizing peptides. HLA-A, -B, and -DR typing were performed by the Puget Sound Blood Center (Seattle, WA).

The patients described in this initial report all had stage IV breast or ovarian cancer. Patients LR3530, KK8162, and TS5437 progressed disease to the point of requiring cytoreductive chemotherapy after receiving the third, second, and third vaccines respectively, and withdrew from the protocol. DG4965 withdrew from the study after the third vaccine for personal reasons and ER9519 developed a systemic infection from an indwelling catheter and withdrew from the study after her second immunization. CC7062, CZ8474, and VC9421 have completed vaccinations or are presently still enrolled.

HER-2/*neu* Peptide-based Vaccines. Peptides were constructed by Multiple Peptide Systems (San Diego, CA). The three peptides included in the ECD vaccine are p42–56, p98–114, p328–345 and in the ICD vaccine are p776–790, p927–941, and p1166–1180. Additional peptides, p369–384, p688–703 and p971–984 had also been defined as potential helper epitopes (7). These were included in the assays as control peptides because they were not included in the vaccines used for these patients. The peptides are solubilized in a 10-mM sodium acetate buffer (pH 4.0). The total vaccine dose administered is 500 μg/peptide for a total dose of 1.5 mg in 0.8 ml using 2 injections of 0.4 ml each. Inoculations were given within the same draining lymph node site and were placed within 5 cm of each other. Each patient was immunized i.d. with a mixture of HER-2/*neu* peptides and GM-CSF (125 μg) kindly supplied by Immunex Corp. (Seattle, WA). Vaccinations were given in the same location monthly.

Detection of T-Cell Responses. Two T-cell evaluations are shown for each patient: (*a*) one evaluation before initiating the study (preimmunization); and (*b*) the final or most recent T-cell evaluation. HER-2/*neu*-specific T-cell responses were measured 30 days after each vaccination, before the next immunization. T-cell proliferation was assessed using a modified limiting dilution assay designed for detecting low-frequency lymphocyte precursors based on Poisson distribution (10). This assay is a modification of a standard limiting dilution analysis (11). The method allows a limited amount of PBMCs to be used to determine a semiquantitative increase in responding T cells sequentially over time, as measured by the number of positive wells. This assay, performed in 24-well replicates rather than the standard 3-well replicates, also allows the calculation of a standard SI, defined as the mean of the response of the antigen-stimulated cells divided by the mean of the response of cells cultured without antigen. SI has proved useful in measuring immunized responses; however, a SI may not adequately reflect the presence of low-frequency responder T cells. The frequency of low numbers of responder cells is more accurately represented by a Poisson distribution of responders, rather than by a normal distribution (10). Therefore, the number of positive wells—determined as the number of wells of test antigen with [^{3}H]thymidine uptake greater than the mean and three SDs of the no-antigen control wells—is also recorded as a measure of low-frequency responses. A cutoff value of the mean and three

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SDs of the 24 no-antigen wells was used to score an experimental well as responding or nonresponding. PBMCs were isolated from heparinized peripheral blood by Ficoll/Hypaque-density gradient centrifugation. All of the HER-2/*neu* peptide and protein antigens were set up in 24-well replicates using 2×10^5 PBMCs/well plated into 96-well round-bottomed microtiter plates obtained from Corning (Corning, NY) in media consisting of equal parts of EHAA 120 (Biofluids) and RPMI 1640 (Life Technologies, Inc.) with L-glutamine, penicillin/streptomycin, 2 ME, and 10% AB serum (ICN Flow, Costa Mesa, CA). Cells were incubated with 25 μg/ml of the various HER-2/*neu* peptides or recombinant HER-2/*neu* domain proteins (ECD/ICD) at several concentrations. Results are shown at protein concentrations of 0.5 μg/ml. After 5 days, wells were pulsed with 1 μCi of [^{3}H]thymidine for 8–10 h and counted. Responses are quantified as the number of responding wells of 24-well replicates. Data are expressed not only as the number of positive wells but also as a standard SI that is defined as the mean of all of the 24 experimental wells divided by the mean of the control wells (no antigen). Ovalbumin, incubated with patient T cells at similar concentrations was used as a negative control protein and in all of the cases showed no response above baseline (data not shown).

Determination of DTH Responses. DTH responses secondary to the i.d. injected vaccines developed in most patients. Responses were measured 48 h after the vaccination. At the end of their series of immunizations, the patients were skin-tested against their individual immunizing peptides as well as the control antigen. Data derived from two patients who have completed all of the six vaccines is presented. One hundred μg of each individual peptide was injected i.d. on the patient's back. As controls, 100 ml of sterile water and 100 μg of GM-CSF were also administered i.d. at a separate location on the back. Induration was measured in mm at 48 h. Up to two DTH sites were biopsied with a 4-mm punch biopsy/patient. Phenopath (Seattle, WA) performed histopathological evaluation of the skin biopsies for CD3, CD4, CD8, CD19, CD1a, and HLA-DR.

RESULTS

Patients Immunized with HER-2/*neu* ECD Peptides Develop HER-2/*neu* Peptide- and Protein-specific T-Cell Responses. The first eight sequential patients that were entered into this trial are included in this interim report. To date, four patients have received two or more immunizations with the ECD vaccine. No patient had evidence of a HER-2/*neu*-specific T-cell response before the immunization (Fig. 1). All of the patients developed peptide-specific responses after at least two vaccine cycles. Three of the four developed HER-2/*neu* protein-specific T-cell responses.

p98 was the dominant peptide epitope eliciting a T-cell response in patient VC9421 (Fig. 1*A*). Twenty-four of 24 wells were positive using modified LDA techniques. The calculated SI to p98 was 14.8. In addition, the patient also developed a response to p42 (24 of 24 wells positive; SI, 5.6). She did not respond to p328. The ECD peptide-specific responses translated into the development of a HER-2/*neu* ECD protein-specific response (SI, 5.5). The response to ECD protein was almost the same level as that detected to the recall antigen tetanus toxoid (SI, 5.7; Fig. 1*A*). During the course of immunization, patient VC9421 also developed T-cell immunity to epitopes not in her immunizing vaccine, which was derived from the ICD. This observed "epitope spreading" resulted in an SI of 3.3 to ICD protein. Patient LR3530 also responded to p98 (SI, 4.0) and p42 (SI, 2.2; Fig. 1*B*). The SI to HER-2/*neu* ECD protein was 6.4. LR3530 also demonstrated epitope spreading with SIs greater than 2.0 to three ICD peptides tested and an SI of 2.2 to ICD protein. Patient KK8162 responded to p42 after two immunizations (SI, 2.7; Fig. 1*C*). The SI to HER-2/*neu* ECD protein was 2.0.

A major concern for the use of peptide-based vaccines is the possibility of developing peptide-specific immunity with no response to native protein. All three of the immunizing ECD peptides—p42, p98, and p328—elicited T-cell proliferation in CZ8474 (Fig. 1*D*). Despite these vigorous peptide-specific T-cell responses, no protein-specific proliferation could be detected to either the ECD or the ICD proteins in this patient.

Patients Immunized with HER-2/*neu* ICD Peptides Develop HER-2/*neu* Peptide- and Protein-specific T-Cell Responses. Four patients had received two or more immunizations with the ICD peptide vaccine. All of the four developed peptide-specific responses (Fig. 2). Three of the four developed protein-specific responses. Patient TS5437 responded to p1166 (SI, 2.2) and to p927 (SI, 2.1; Fig. 2*A*). The SI to the ICD protein was 2.3. Patient TS5437 also demonstrated epitope spreading to peptides not in her immunizing mix, including p971 from the ICD and p328 and p369 from the ECD as well as response to the ECD protein (SI, 2.1). ER9519 was the only patient studied who had a preexisting immune response to the HER-2/*neu* protein (ECD SI of 5.2; Fig. 2*B*). By chance, patient ER9519 randomized to receive the ICD vaccine. After two immunizations, the patient had developed T-cell responses to p927 (SI, 2.5), a peptide in her immunizing mix, as well as to peptides not in the vaccine including p971 (SI, 2.2) and p688 (SI, 2.1). p971 is from the ICD. p688 is located in the transmembrane domain. The response to the ICD protein, not detectable before immunization, was SI of 2.6. Patient DG4965 developed T-cell responses to p927 (SI, 3.5) in the vaccine and to a closely positioned peptide not in the vaccine—p971 (SI, 6.4; Fig. 2*C*). The response to HER-2/*neu* ICD protein was SI 5.9. Patient DG4965 demonstrated epitope spreading with significant T-cell proliferation to p328 and p369—ECD peptides not in her immunizing mix. Of note, although no ECD-specific immunity was present before immunization, she had an SI of 3.4 to the ECD protein after three ICD peptide vaccine administrations.

Peptide-specific T-cell responses, without evidence of recognition of the HER-2/*neu* protein, developed in one patient immunized with the ICD peptide vaccine. CC7062, the first patient to complete all of the six inoculations, developed T-cell immune responses to two of her immunizing peptides, p776 (SI, 4.6) and p927 (SI, 2.2) but no response to ICD protein or ECD protein (Fig. 2*D*).

To date, there is no significant correlation with any peptide-specific response to a particular HLA type. The HLA-A, -B, and -DR of these patients are shown in Table 1.

HER-2/*neu* Peptide Immunization Elicits Epitope Spreading. Patients in both the ECD peptide- or ICD peptide-immunized groups demonstrate epitope spreading, that is, re-

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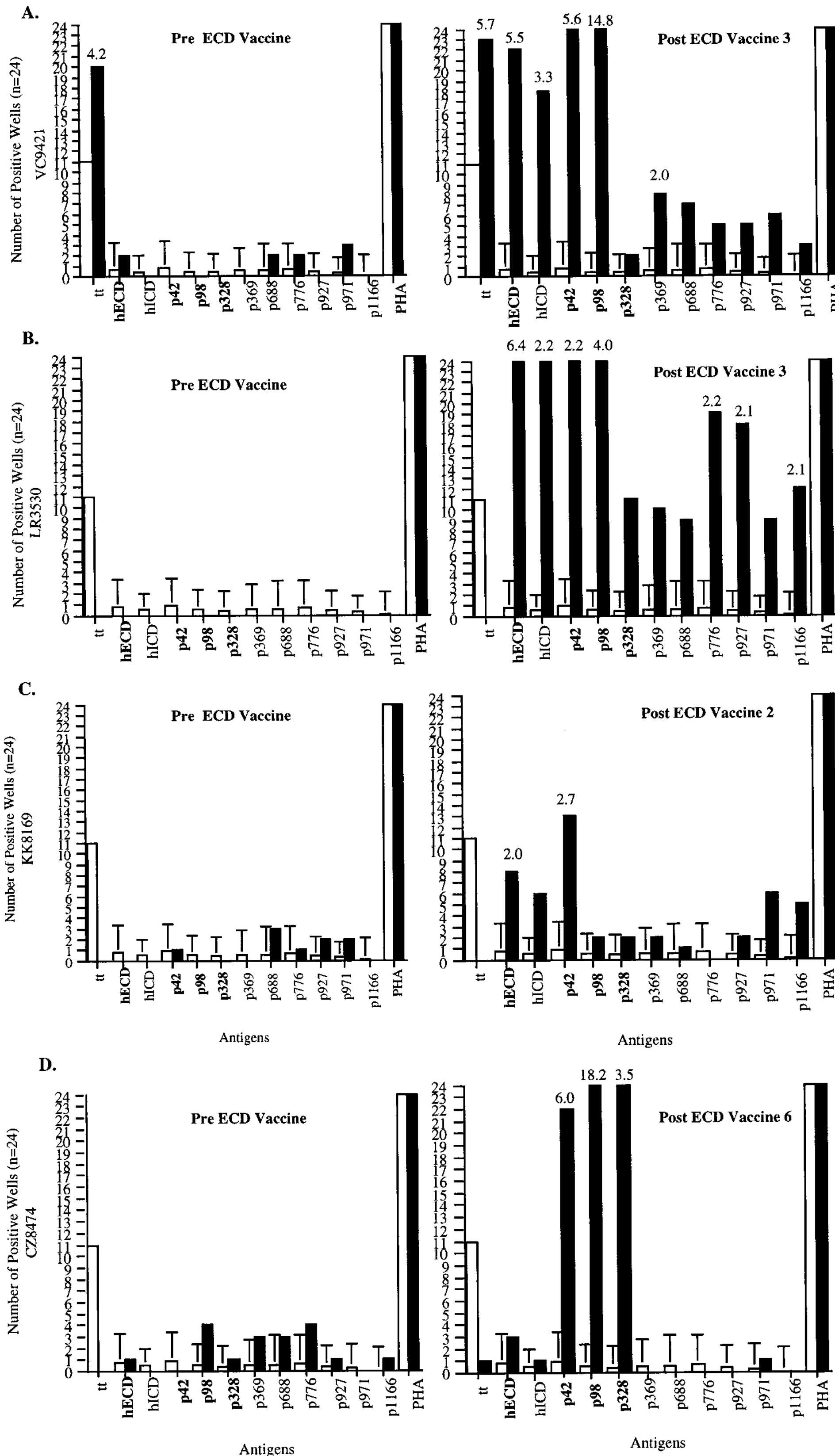

Fig. 1 Patients immunized with HER-2/*neu* ECD peptides develop HER-2/*neu* peptide and protein-specific T-cell responses. Data are shown from four patients immunized with the ECD peptide vaccine: *A*, VC9421; *B*, LR3530; *C*, KK8162; and *D*, CZ8474. HER-2/*neu* antigens were tested in 24-well replicates, and wells scored positive if the cpm was greater than the mean and three SDs of the 24 no-antigen wells. The three peptides included in the vaccine formulation are in **bold** type. T-cell responses to individual peptides and to ICD and ECD proteins are displayed as the *number of positive wells*. □, the mean and two SDs of the response in normal blood donors (n = 20). ■, the HER-2/*neu*-specific T-cell response before and after immunization. *Numbers above the antigen columns*, calculated SIs (the mean of all of the 24 experimental wells divided by the mean of 24 no-antigen wells) when the SI was ≥2.0. Phytohemagglutinin is included in each assay as a positive control for T-cell proliferation, and tetanus toxoid (tt) as a control recall antigen, although some patients had not been vaccinated for many years.

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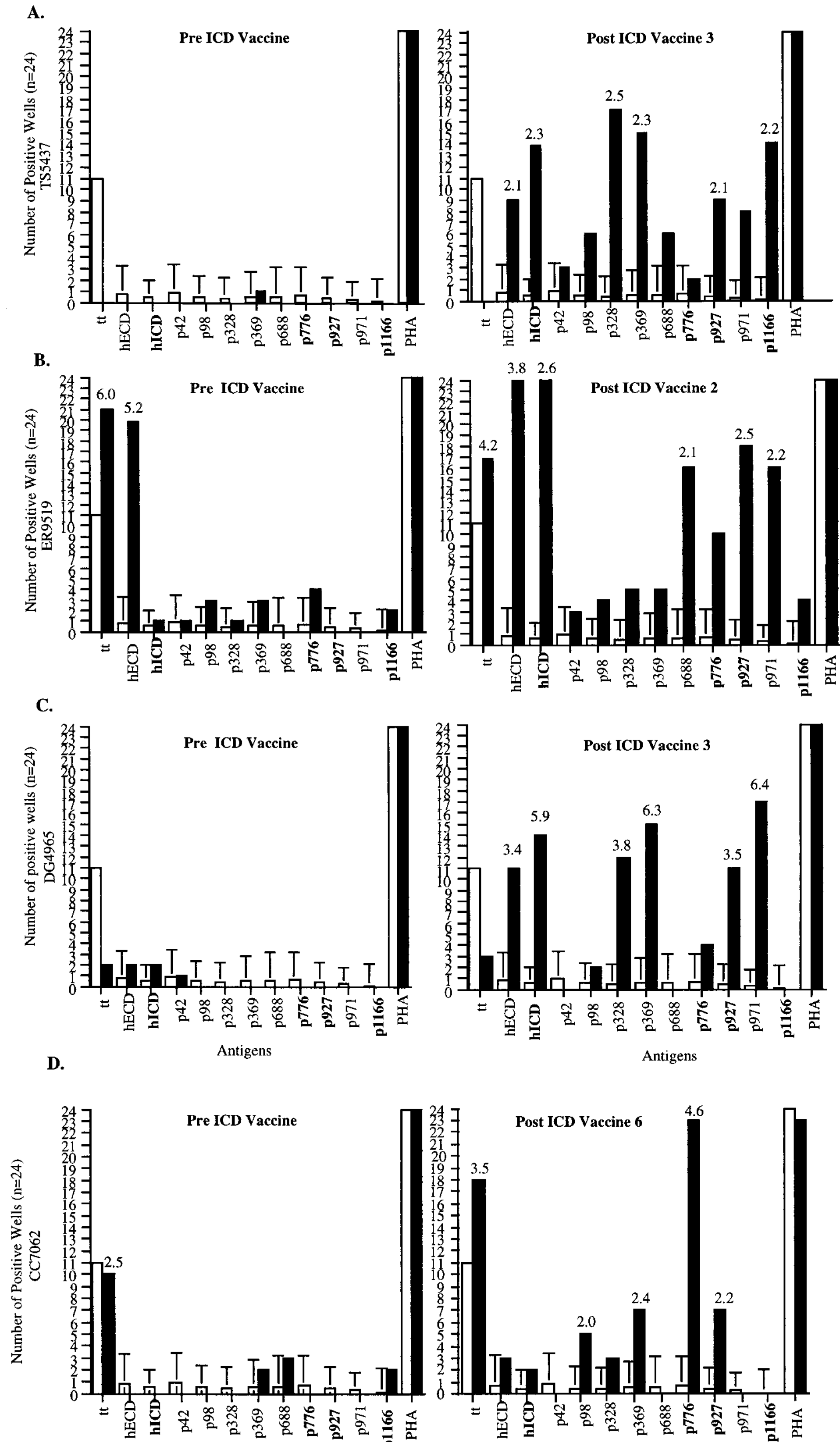

Fig. 2 Patients immunized with HER-2/*neu* ICD peptides develop HER-2/*neu* peptide and protein-specific T-cell responses. Data are shown from four patients immunized with the ICD peptide vaccine: *A*, TS5437; *B*, ER9519; *C*, DG4965; and *D*, CC7062. The three peptides included in the vaccine formulation are in *bold* type. T-cell responses are displayed as the *number of positive wells*. □, the mean and two SDs of the response in normal blood donors ($n = 20$). ■, the HER-2/*neu*-specific T-cell response before and after immunization. *Numbers above the antigen columns*, calculated SIs (the mean of all of the 24 experimental wells divided by the mean of 24 no-antigen wells) when the SI was ≥2.0.

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Table 1 HLA type of patients enrolled in study

HLA-A, -B, and -DR were determined on aliquots of PBMCs at the time of entry into the study.

Patient	HLA-A	HLA-B	HLA-DR
VC9421	24,26	14,45	ND[a]
LR3530	1	8	3,11
KK8169	3,24	35,51	ND*
CZ8474	24	49,62	11,13
TS5437	1,28	37,62	4,10
ER9519	3,29	35,44	1,7
DG4965	1,23	7	1,10
CC7062	1,2	27	1,11

[a] ND, not determined. Inadequate number of lymphocytes available.

Table 2 Patients develop DTH at the vaccination site

DTH responses were measured and recorded 48 h after each vaccine administration.

	Induration (mm) at 48 h	
Patients	DTH: vaccine 1	DTH: recent vaccine
ECD		
VC9421	None	Confluent 80 × 64
LR3530	None	23/22
KK8169	None	Not available[a]
CZ8474	None	Confluent 60 × 75
ICD		
TS5437	None	10/14
ER9519	Red, warm. No induration.	20/16
DG4965	None	22/18
CC7062	None	Confluent 48 × 59

[a] Patient reported "small lump" in inoculation site, not measured.

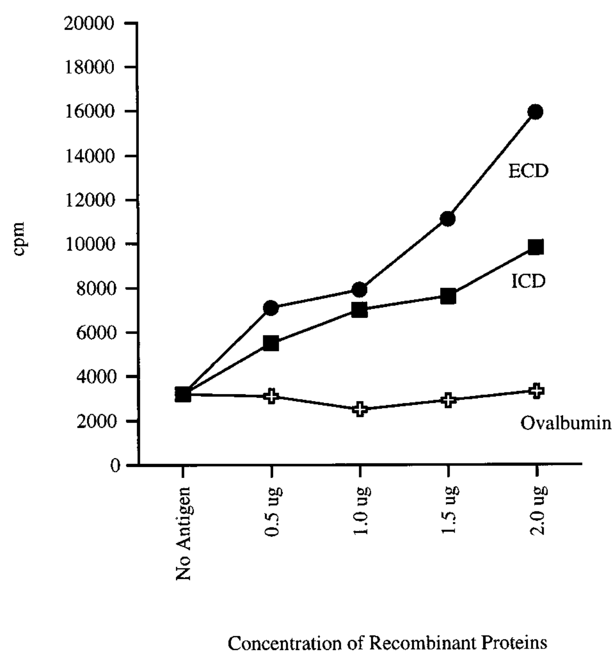

Fig. 3 HER-2/*neu* peptide immunization elicits epitope spreading. Recombinant human ECD and ICD proteins were evaluated in 24-well replicates at four concentrations. Data are expressed as the mean and SD cpm of the 24-well replicates for the human HER-2/*neu* ECD or ICD protein. The patient demonstrated no response above baseline to ovalbumin, which was used as a negative control protein.

sponding to portions of the HER-2/*neu* protein not included in their immunizing mix. Fig. 3 represents the T-cell response curves to both the ECD and the ICD recombinant proteins in a patient immunized with the ECD peptide vaccine, VC9421, as an example of this observation. In a concentration-dependent fashion the SI is increased to both the ECD and the ICD proteins, although the patient was immunized with ECD peptides only. The patient had no T-cell response above baseline to ovalbumin.

T-Cell Responses *in Vitro* Correlate with Peptide-specific DTH Responses *in Vivo*. The development of DTH in response to the vaccination formulation (groups of peptides plus GM-CSF) was evaluated after each immunization. After the sixth immunization, the DTH to individual peptides was determined. Because of the volume of the vaccine (800 ml), the immunizations were given in two 400-ml i.d. injections within a single draining lymph node region (within 5 cm of each other). After the first immunization, no DTH was noted in any patient (Table 2). After multiple immunizations, DTH developed at the vaccination site in all but one patient (KK8169), and that patient had had only two immunizations. In three patients (VC9421, CZ8474, and CC7062), the DTH responses were so vigorous that the two individual vaccination sites became confluent and were measured as a whole. All of the DTH responses to the vaccine were over 10-mm^2 induration.

Data derived from two patients who have completed all of the six immunizations [CZ8474 (ECD vaccine) and CC7062 (ICD vaccine)] are included in this initial report. These patients underwent skin testing to the peptide components of their vaccine and to GM-CSF separately. Patient CZ8474 had DTH responses (Table 3A) and proliferative responses (Fig. 1*D*) to the three peptides contained in her vaccine formulation (p42, p98, and p328). Histological evaluation of the biopsy site of p98 revealed marked lymphocytic infiltration at the dermal border (data not shown). The infiltrate was predominantly CD3+ and CD4+. The results were similar to those for patient CC7062 (Fig. 4). No DTH or proliferative response occurred to p971, a peptide that was not in her vaccine formulation. There was no response to GM-CSF by DTH; however, the patient had a detectable peripheral blood T-cell response to GM-CSF.

Patient CC7062 had induration >5 mm^2 to the two peptides in the vaccine formulation that induce T-cell proliferative responses, p776 and p927 (Table 3B). Histological evaluation of a skin biopsy of p776 revealed marked lymphocytic infiltration at the dermal border (Fig. 4). The infiltrate was predominantly CD4+. There was an increase in CD1a and HLA-DR positive cells. There was a minimal DTH response and no T-cell proliferative response to the third peptide in the vaccine formulation, p1166. A significant DTH response occurred to p98, an ECD epitope to which the patient had detectable T-cell response *in vitro*. There was no response to GM-CSF.

This initial report includes a limited number of patients; however, although detectable HER-2/*neu*-specific immunity was generated with this immunization strategy, no toxicity was

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Table 3 T-cell responses *in vitro* correlate with peptide-specific DTH responses *in vivo*

Two patients completed all of the six immunizations and were skin-tested to the peptide components of their vaccine and GM-CSF individually. A, patient CZ8474. Skin test was performed 1 month after the sixth and final ECD vaccination. One-hundred μg of each HER-2/*neu* peptide was placed separately from 100 μg of GM-CSF i.d., and induration was measured at 48 h. B, patient CC7062. Skin test was performed 1 month after the sixth and final ICD vaccination. One-hundred μg of HER-2/*neu* peptides were placed separately from 100 μg of GM-CSF i.d., and induration was measured at 48 h.

Antigen tested	Induration (mm) 48 h
A. CZ8474	
p42	15 × 12
p98	12 × 12
p328	8 × 10
p971	0
Sterile water	0
GM-CSF	0
B. CC7062	
p776	11 × 11
p927	10 × 10
p1166	4 × 3
p98	6 × 6
Sterile water	0
GM-CSF	0

noted against tissues that express basal levels of the HER2-/*neu* protein, specifically skin, digestive tract epithelium, lung, and liver. All of the patients were monitored monthly with a complete blood count, serum chemistries that included liver and renal function tests, and electrolytes. No changes from baseline were noted in any of the patients. In addition, a complete physical examination was performed on each patient before each immunization. No physical abnormalities resulted during the course of immunization. In this initial report, only one toxicity has been noted. Patient CZ8474 developed urticaria and generalized puritis associated with her final vaccine and subsequent skin tests (12). Her symptoms resolved over 30 min after the administration of oral diphenhydramine in both instances.

DISCUSSION

The vaccination strategy reported here—immunization with HER-2/*neu*-derived peptides in GM-CSF as an adjuvant—was highly effective in generating HER-2/*neu* protein-specific T-cell immunity. In general, studies evaluating peptide-based cancer vaccines have not demonstrated detectable protein-specific immunity, or even high-level peptide-specific T-cell responses (13). The critical differences between previous studies and the present study may be the antigen system, the type of T-cell response elicited, the adjuvant, or the route of immunization.

The peptide epitopes chosen for the present study were highly selected. Potential "subdominant" HER-2/*neu* peptides were chosen based on motif, extensive *in vitro* evaluation, and the assessment of existent immune responses to HER-2/*neu*, which have been noted in some patients with breast cancer. 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 those related to amphipathic α helices (14). Although peptides that bind to class II molecules are now known not to necessarily form α helical orientations, each of the searching algorithms had empirically been successful in identifying a substantial proportion (50–70%) of helper T-cell epitopes in foreign proteins (15, 16). The analysis resulted in the identification of more than 40 HER-2/*neu* peptides with a high potential for interacting with human class II molecules (17, 18). On the basis of predicted interactions, 26 peptides, 15–18 amino acids in length, were constructed. Seven of 26 peptides that were tested demonstrated the ability to elicit T-cell responses *in vitro* in at least some of the breast cancer patients evaluated (7). Six of the seven peptides were used in the present study.

The vaccine consisted of three peptides derived from the ECD or three peptides derived from the ICD. Peptides derived from the different domains were tested separately because we hypothesized that the two domains of the HER-2/*neu* protein might behave differently immunologically. In theory, the ICD may be more immunogenic. As an intracellular protein, it is sequestered and not readily available for immune recognition and, thus, tolerance induction. By contrast, the ECD protein is shed and circulates in the sera of some patients as a soluble protein, therefore, is available to the immune system for tolerance induction (19). Despite the theory, to date, the immune responses detected to the ICD peptides and ECD peptides seem to be equivalent.

Our vaccine strategy used an i.d. injection of GM-CSF with peptide in an attempt to stimulate DC recruitment and maturation *in vivo*. GM-CSF has been shown *in vitro* to stimulate the growth of potent dendritic and macrophage APCs (20, 21). DCs normally reside as Langerhans cells in the dermis. Administered i.d., GM-CSF induces DCs locally in humans (6) and results in the trafficking of class II-positive cells from skin to draining lymph nodes in rats (5). In prior studies in rats, evaluating soluble GM-CSF as an adjuvant, i.d. administration of the cytokine with rat *neu* peptides boosted *neu*-specific T-cell immunity (5). Studies shown here indicate that GM-CSF injected i.d. is an effective adjuvant in humans for use in HER-2/*neu* peptide-based vaccines.

Six of eight patients immunized with peptides developed both peptide- and protein-specific T-cell responses as defined by a SI >2.0. The detection of protein-specific T cells after peptide immunization implies that one or more of the immunizing peptides represent natural epitopes of the HER-2/*neu* protein. The generation of HER-2/*neu* protein-specific responses, in this study, was associated with epitope or determinant spreading. This phenomenon, first described in autoimmune disease (22), suggests that the immune repertoire to HER-2/*neu* evolves during the course of vaccination. The development of a T-cell response with a variety of specificities during immunization indicates that naturally expressed HER-2/*neu* protein is being processed and presented in an augmented fashion. Whether this phenomenon results from more efficient APCs induced by GM-CSF, the generation of

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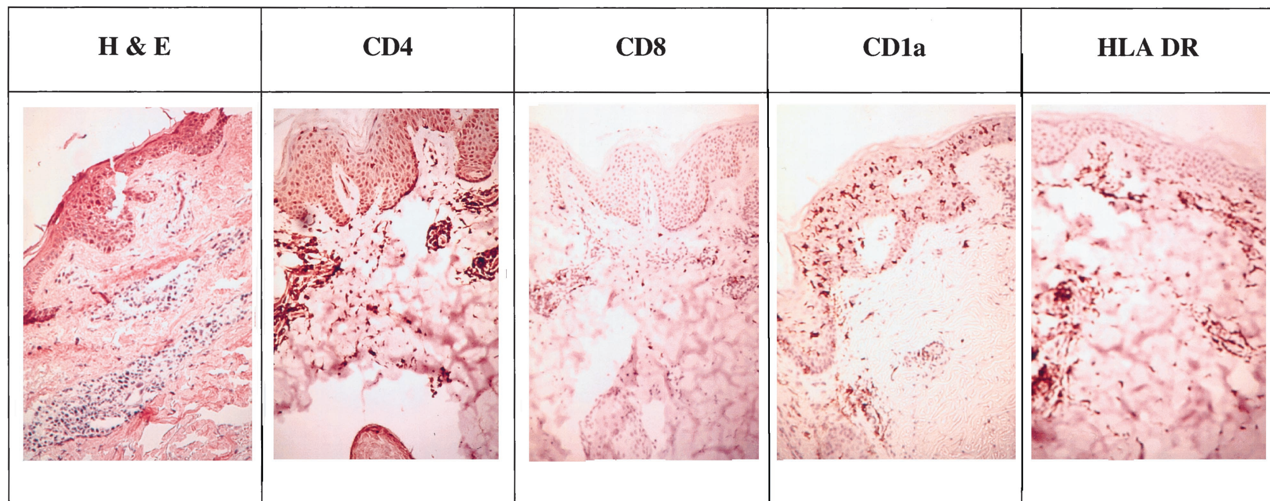

Fig. 4 The DTH response after immunization is a predominant CD4+ T-cell infiltrate. H&E staining of the p776-induced dermal cellular infiltrate. CD4, CD8, CD1a, and HLA-DR—derived from the same biopsy sample—are also shown. ×14.

an antibody response that concentrates antigens into APCs or a more appropriate cytokine environment is unknown (23). Epitope spreading has been associated with an antigen-specific Th1 response in some models (24). Studies to determine the cytokine secretion pattern of the T cells elicited through HER-2/*neu* peptide vaccination are presently underway.

A paradox of tumor immunology is the observation that some patients have existing tumor-specific T-cell immunity in the face of a growing cancer. Clearly, the endogenous immune response detected in cancer patients directed against their own tumors is not adequate. A driving hypothesis in cancer vaccine development is that cancer immunity that results from immunization by the patient's own tumor is not amplified but is rather of very low magnitude. Indeed, if one could boost cancer-specific T-cell immunity via vaccination, perhaps then, tumor could be eradicated. i.d. immunization of HER-2/*neu* peptides with GM-CSF can result in significant T-cell responses, in some cases to the level of a vaccinated foreign antigen such as tetanus. Two of the patients in this study had detectable immunity to tetanus protein after a recent vaccination within the last 5 years, and the level of T-cell immunity to HER-2/*neu* protein approached or was equivalent to that of tetanus after peptide immunization.

In addition, investigations described here imply that HER-2/*neu*-specific T cells can migrate outside the peripheral circulation. Although all of the patients developed DTH responses to their vaccines at the initial site of injection, the ability to mediate specific DTH at distant sites was validated in the two patients who had completed the vaccine regimen. Biopsies of the positive DTH sites revealed classic DTH with a marked lymphocytic infiltrate at the dermal junction. The T-cell infiltrate was predominantly CD4+. DTH testing to only a limited number of peptides was allowed by protocol. Thus, more extensive correlative studies were not performed. However, results to date show a correlation between T-cell responses *in vitro* and peptide-specific DTH responses *in vivo*. Thus, HER-2/*neu* peptide vaccines can result in the generation of T cells that have the ability to "home" to antigen *in vivo*.

One problem for the use of peptide immunization noted from studies attempting to prime *in vitro* or prime *in vivo* in animal models is that often the elicited peptide-specific T cells do not respond to protein. In the present study, two of eight patients developed peptide-specific responses with no evidence of protein-specific responses. Presumably the lack of protein responses relate to particular MHC restriction elements; however, both class I and class II HLA typing have been performed on all of the patients enrolled in the study and, to date, there are no predominant MHC molecules that predict responses. It is unlikely that peptide-specific responses without protein-specific responses can translate to a therapeutic effect. Thus, although the majority of the patients (six of eight) developed protein-specific responses, some matching of peptides to particular MHC molecules may be required to elicit protein responses in all patients.

Peptide-based vaccines offer many advantages to protein- or tumor-based vaccines such as ease of construction, chemical stability, and the lack of oncogenic or infectious material. The major drawbacks for peptide-based vaccines are that peptides are considered to be weakly immunogenic, and standard methods of peptide immunization in humans have not been defined. Also, the disparate HLA type of an outbred human population may not allow peptide vaccines to be widely applicable to all patients. The present study showing responses to HER-2/*neu* protein in six of the first eight patients immunized by using a very simply constructed vaccine provides encouragement for testing responses in other cancer antigen systems. Although the majority of patients developed HER-2/*neu* protein-specific T-cell responses, enrollment continues to determine whether this observation can be extrapolated to a large number of patients. Finally, studies are under development to determine whether the generation of immunity to HER-2/*neu* translates to an anticancer effect.

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