

Delayed-Type Hypersensitivity Response Is a Predictor of Peripheral Blood T-Cell Immunity after HER-2/neu Peptide Immunization¹

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ABSTRACT

Many groups that immunize cancer patients with cancer vaccines use the generation of a delayed-type hypersensitivity (DTH) response as the primary measure of the ability to immunize a patient to a tumor cell or specific tumor antigen. This study examines whether the development of a tumor antigen-specific DTH response, measured after vaccination with peptide-based vaccines, correlates to *in vitro* assessment of peripheral blood antigen-specific T-cell responses. The HER-2/neu protein was used as a model tumor antigen. Thirty-two patients who completed a course of immunization with HER-2/neu peptide-based vaccines were analyzed. HER-2/neu peptide-specific DTH responses ($n = 93$) and peripheral blood T-cell responses ($n = 93$) were measured 30 days after the final immunization. Size of DTH induration was correlated with HER-2/neu-specific T-cell proliferative responses assessed from peripheral blood lymphocytes isolated concurrently with peptide skin test placement. HER-2/neu peptide-specific DTH responses ≥ 10 mm² correlated significantly to a measurable peptide-specific peripheral blood T-cell response defined as stimulation index > 2.0 ($P = 0.0006$). However, antigen-specific DTH responses with magnitudes between 5 and 9 mm² were not significantly associated with the development of systemic immunity. DTH responses between 5 and 9 mm² carried an odds ratio of 1.3 ($P = 0.61$) in predicting a measurable systemic tumor antigen response. The findings presented here demonstrate that tumor antigen-specific DTH re-

sponses ≥ 10 mm² correlate with measurable *in vitro* antigen-specific lymphocytic proliferation and are, in this model system, a reflection of systemic immunization.

INTRODUCTION

The proposed end point of many initial studies of cancer vaccines is the ability to generate an immune response against a cancer antigen or the correlation of an antitumor response to some measure of immunity. At present, there is no standard methodology for measuring cell-mediated immune responses elicited by cancer vaccines. Recent advances in molecular tumor immunology have resulted in the identification of several specific tumor antigens defined by virtue of their immunogenicity in cancer patients (1, 2). Immunizing against a specific tumor antigen allows the comparison of methods to measure tumor-specific immunity and a statistical assessment of the validity of various means of cancer vaccine immunological monitoring. DTH³ responses are the most common, most widely available measurements, and one of the few measurements used to determine effective tumor immunization. However, little is known about the ability of DTH to actually reflect the development of systemic tumor-specific immunity in cancer patients after vaccination.

Our group has been studying the HER-2/neu protein as a tumor antigen. Early reports of a phase I study of HER-2/neu peptide-based vaccines indicate that measurable systemic immunity to HER-2/neu peptides, and to the protein itself, can be generated after peptide immunization (3). The systemic immune response was assessed by *in vitro* evaluation of lymphocytic proliferation. The present study questions whether tumor antigen-specific DTH responses, elicited after vaccination, are an adequate reflection of the generation of systemic cell-mediated immunity. In addition, this study examines what the magnitude of the tumor antigen DTH response must be to potentially predict the acquisition of systemic antigen-specific immunity.

PATIENTS AND METHODS

Patient Population. The University of Washington Human Subjects Division and the United States Food and Drug Administration approved a phase I trial of HER-2/neu peptide vaccines. Patients with stage III or IV breast, ovarian, or non-small cell lung cancer were eligible for study if the following criteria were met: (a) HER-2/neu protein overexpression in the primary tumor or metastasis had been documented; (b) the patient had received prior treatment and had, at entry, either no detectable cancer or minimal residual disease stable on hormonal or radiotherapy; (c) the patient had the clinical expectation to

Received 7/12/99; revised 12/20/99; accepted 1/14/00.

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¹ Supported by NIH Grant P32 CA09515-14 (to D. G. M. and K. R.), and by grants from NIH, the National Cancer Institute (Grants K08 CA61834 and R01 CA75163 to M. L. D.), the Cancer Research Treatment Foundation, and the Department of Defense Breast Cancer Program. Patient care was conducted through the Clinical Research Center Facility at the University of Washington, which is supported through NIH Grant MO1-RR-00037.

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³ The abbreviations used are: DTH, delayed-type hypersensitivity; i.d., interdermal; PBMC, peripheral blood mononuclear cell; OR, odds ratio; CI, confidence interval; SI, stimulation index.

Table 1 Characteristics of the study population

The age range, location, and disease status of HER-2/neu-overexpressing cancers present in the patients evaluated are shown.

Diagnosis	Disease status			Total no. of patients (n = 32)
	Complete response	Stable disease	Progressive disease	
Breast	14	11	1	26
Stage III	9	NA ^a	NA	9
Stage IV	5	11	1	17
Ovarian	2	1	1	4
Stage III	1	1		2
Stage IV	1		1	2
NSC lung	1			2
Stage III	1			1
Stage IV		1		1
Age, median years (range)	51 (34–86)			

^a NA, not applicable; NSC, non-small cell.

remain off any immunosuppressive therapy for 6 months; and (d) the patient has signed an informed consent. Patients received a vaccine composed of three peptides derived from the HER-2/neu protein. Three vaccine formulations were evaluated, each containing three different peptides (3). Subjects were immunized monthly for 6 months. At the end of six immunizations, patients were skin tested against individual immunizing peptides, and DTH responses were measured. Peripheral blood was obtained at the time of skin test placement to measure antigen-specific peripheral T-cell responses. The data presented here reflect 32 consecutive patients who met entry criteria, completed six immunizations, and received individual peptide skin tests.

HER-2/neu Peptide-based Vaccines and Skin Tests.

Peptides were constructed by Multiple Peptide Systems (San Diego, CA). All peptides constructed were potential helper epitopes of the HER-2/neu protein predicted by computer modeling and empiric testing to be potentially immunogenic (4). Three vaccine formulations were tested. The first vaccine contained peptides p42-56, p98-114, and p328-345; nine patients received this vaccine. The second vaccine included peptides p776-790, p927-941, and p1166-1180; nine patients received this vaccine. The final vaccine formulation consisted of peptides p369-384, p688-703, and p971-984; 14 patients received this vaccine. The peptides were solubilized in a 10 mM sodium acetate buffer (pH 4.0). The total vaccine dose administered was 500 µg per peptide for a total dose of 1.5 mg in 0.8 ml using two injections of 0.4 ml. Inoculations were given in the same location each month within the same draining lymph node site and were placed within 5 cm of each other. Each patient received i.d. immunizations containing a mixture of HER-2/neu peptides and 125 µg of recombinant human granulocyte macrophage colony-stimulating factor (Immunex Corporation, Seattle, WA). Each peptide was also packaged individually in vials for subsequent skin testing at the completion of the study (Corixa Corporation, Seattle, WA).

Determination of DTH Responses. Patients were skin tested against their immunizing peptides 1 month after their last vaccination. One hundred µg of each individual peptide were injected i.d. on the patient's back, a site distant from the vaccine site. As a control, 100 µl of sterile water was also administered

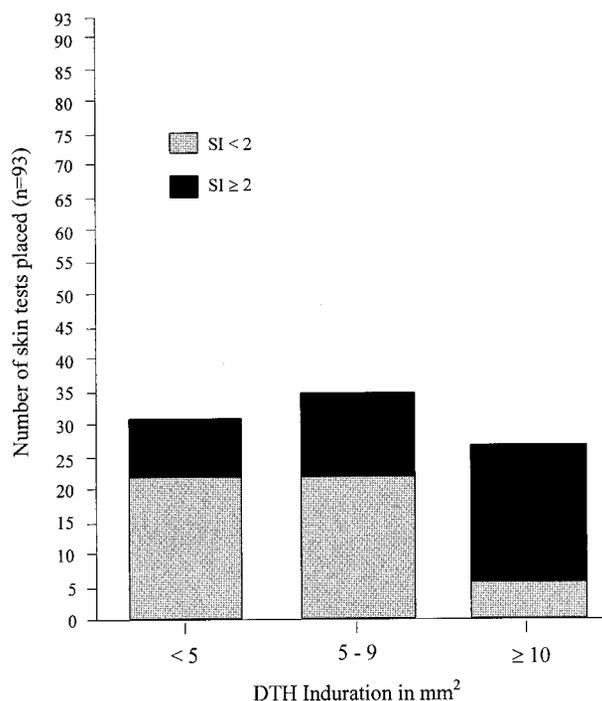


Fig. 1 HER-2/neu peptide-specific DTH responses ≥ 10 mm² correlate significantly to antigen-specific lymphocyte proliferation *in vitro*. Results from 93 HER-2/neu peptide skin tests are compared with a matched *in vitro* evaluation of HER-2/neu peptide-specific T-cell response after HER-2/neu peptide immunization. DTH responses are categorized as ≥ 10 mm² induration, 5–9 mm², and < 5 mm². These results are compared with peripheral blood HER-2/neu peptide-specific lymphocyte responses with SI ≥ 2.0 or < 2.0 as a measure of immunization.

i.d. Induration was measured using calipers and reported in mm across two diameters at 48 h. Skin tests were read by one of two research nurses trained in DTH assessment. The clinical staff was blinded to the results of the laboratory tests and *vice versa* because the analysis of skin and peripheral blood responses was performed simultaneously.

Table 2 Antigen-specific DTH responses are not significantly associated with measurable systemic immune responses

The ORs and *P* values for DTH 5–9 mm² and ≥10 mm² associated with SI ≥2.0 are shown.

DTH (mm ²)	OR	<i>P</i>
<5	1.0	
5–9	1.3	0.61
≥10	8.2	0.0006

Detection of Peripheral Blood T-Cell Responses.

HER-2/neu peptide-specific T-cell responses were measured at baseline and at 30 days after each vaccination, prior to the next immunization. Peripheral blood mononuclear cells were prepared by Ficoll-Paque (Pharmacia AB, Uppsala, Sweden) centrifugation and resuspended in medium consisting of equal parts of EHAA 120 (Biofluids Inc., Rockville, MD) and RPMI 1640 (Life Technologies, Inc., Grand Island, NY) with L-glutamine, penicillin/streptomycin, symbol 98 -2ME, and 10% AB serum (ICN Flow, Costa Mesa, CA). PBMCs were cocultured with 50 μg/ml of the various individual HER-2/neu peptides. Specifically, 2 × 10⁵ PBMCs/well were plated into 96-well round-bottomed microtiter plates (Costar, Cambridge, MA) with antigen at 37°C in an atmosphere of 5% CO₂ for 5 days. All antigens were tested in 24-well replicates. Eight h before termination of culture, each well was pulsed with 1 μCi of [³H]thymidine (New England Nuclear, Wilmington, DE). The cultures were then harvested onto glass fiber filters, and the incorporated radioactivity was measured with a Microbeta 1450 scintillation counter (Wallac, Turku, Finland). Peripheral blood T-cell response data presented here are expressed as a standard SI, which is defined as the mean of all 24 experimental wells divided by the mean of the 24 control wells (no antigen). Phytohemagglutinin, incubated with patient T cells at a concentration of 5 μg/ml, was used as a positive control for the ability of T cells to respond to antigen and produced an SI >2.0 in all patient assays reported (data not shown).

Statistical Methods. The association of postvaccination DTH responses with the probability of a postvaccination antigen-specific SI >2.0 was examined using logistic regression. Adjustments were made in the variance because of the multiple samples taken per patient. An estimate of the OR was obtained by exponentiating the appropriate parameter estimate from the regression model. The associated 95% CI was obtained from the parameter estimate and its adjusted error. The *P* from the regression model is two-sided and was derived using the Wald test.

RESULTS

HER-2/neu Peptide-specific DTH Responses ≥10 mm² Correlate Significantly to Antigen-specific Lymphocyte Proliferation *in Vitro*. Patients immunized with HER-2/neu peptide-based vaccines had advanced stage HER-2/neu-overexpressing breast, ovarian, or non-small cell lung cancers (Table 1). A total of 93 skin tests were placed, and 93 *in vitro* T-cell analyses were performed on 32 patients. DTH tests were categorized as ≥10 mm² (*n* = 27), between 5 and 9 mm² (*n* = 35),

Table 3 Immunizing Significant DTH responses in relation to the immunizing peptide

Immunizing peptides, number of patients tested with each peptide, and number of DTH responses ≥10 mm² are shown.

Immunizing peptide	No. of patients tested	No. of DTH ≥10 mm ²
p42-56	9	5
p98-114	9	3
p328-345	9	1
p369-384	14	7
p688-703	14	3
p776-790	9	3
p927-941	6	2
p971-984	14	2
p1166-1180	9	1

and <5 mm² (*n* = 31). Similarly, the HER-2/neu peptide-specific peripheral blood T-cell responses were defined as an SI ≥2.0 or an SI <2.0. Forty-three HER-2/neu peptide peripheral blood T-cell analyses were associated with an SI ≥2.0. For the majority of patients, the increased SI represented a change from baseline values measured prior to the first immunization (*n* = 41). One patient did not have an SI value for an immunizing peptide at baseline because of a low number of cells available for analysis, and a second patient had an SI of 2.0 at baseline that did not increase after immunization. *In vitro* T-cell responses were evaluated against an irrelevant nonimmunizing peptide in all patients, and no patient demonstrated an increase in SI over time to the irrelevant peptide (all SIs to irrelevant peptide <2.0). Matched skin tests for those assays ≥2.0 demonstrated 21 DTH responses ≥10 mm², 13 DTH responses between 5 and 9 mm², and 9 DTH responses <5 mm² (Fig. 1). Similarly, 50 HER-2/neu peptide peripheral blood T-cell analyses were associated with an SI <2.0. Matched skin tests for those assays demonstrated 6 DTH responses ≥10 mm², 22 DTH responses between 5 and 9 mm², and 22 DTH responses <5 mm² (Fig. 1). The odds of an SI being ≥2.0 for a DTH ≥10 mm² compared with a DTH <5 mm² were significant: OR = 8.2; 95% CI, 2.5–27.0; *P* = 0.0006 (Table 2).

Antigen-specific DTH Responses between 5 and 9 mm² Are not Significantly Associated with Measurable Systemic Immune Responses. The odds of observing an antigen-specific SI ≥2.0 increased as the DTH response increased (*P* = 0.007) when the DTH response was analyzed as a continuous variable. The odds of an SI ≥2.0 were greater for a DTH measuring 5–9 mm² than for a DTH of <5 mm² induration, although the difference was not statistically significant: OR = 1.3; 95% CI, 0.5–3.8; *P* = 0.61 (Table 2). Seven patients had more than one DTH response ≥10 mm². There was no association between a particular peptide and a significant DTH response; however, the limited number of patients and multiple parameters to be evaluated did not allow a rigorous statistical evaluation (Table 3). In addition, the number of patients in each disease and stage category was too small to draw any statistical correlation between disease status and the development of an immune response.

DISCUSSION

Cancer vaccines targeting specific proteins, compared with strategies using various modifications of whole tumor cells, offer an advantage in terms of immunological monitoring. Immunizing against a specific antigen and developing methods to evaluate the resultant immune response allows comparison of different monitoring techniques. *In vitro* assays of cell-mediated immunity directed against various tumor antigens are available in research laboratories. These assays, such as antigen-specific lymphocyte proliferation, require the purification of lymphocytes from the peripheral blood of patients and a quantitative assessment of the blastogenic response to an immunizing antigen. For an infectious disease antigen, such as tetanus toxoid, antigen-specific lymphocyte proliferation has been shown to correlate well with a significant tetanus toxoid DTH response (5). Thus, in infectious disease models, the DTH response, a technically simple test, reflects the development of systemic antigen-specific immunity. Such a relationship has not been demonstrated for tumor-specific immune responses. We questioned whether the development of tumor antigen-specific DTH responses, using HER-2/neu peptides as the model antigen in patients with advanced-stage cancer, correlate to *in vitro* measurements of systemic antigen-specific T-cell responses as measured by lymphocytic proliferation. The data presented here demonstrate that tumor antigen-specific DTH responses ≥ 10 mm² correlate significantly to a measurable antigen-specific peripheral blood T-cell response.

Many cancer vaccine strategies use intact tumor cells or tumor cell lysates; therefore, the potential immunizing antigen(s) are unknown. In these systems, it is very difficult to design *in vitro* T-cell assays that would measure potential immunization. Evaluation of the DTH response to tumor or tumor lysate is the primary method of immunological assessment. The measurement of DTH responses specific for whole tumor preparations has been shown to correlate with improved survival after immunization in patients with melanoma (6, 7). The data described here evaluated local and systemic T-cell immunity to tumor antigen-specific peptides after active immunization. Evaluation of peptide responses serves as a model for comparing the

evaluation of DTH and *in vitro* T-cell responses after immunization in patients with advanced-stage cancer. These results indicate that significant DTH responses, which develop in cancer patients post immunization are, potentially, a reflection of the development of systemic cell mediated immunity.

ACKNOWLEDGMENTS

We are grateful for the expert nursing care of Donna Davis and the technical expertise of Paul Crosby. We thank Immunex Corporation, Seattle, WA, for supplying the recombinant human granulocyte macrophage colony-stimulating factor used in the vaccination of patients, and Corixa Corporation, Seattle, WA for supplying peptide vaccines and DTH tests. We thank Bill Hix for assistance in manuscript preparation.

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Clinical Cancer Research

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Clin Cancer Res 2000;6:1347-1350.

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