Immunization of Cancer Patients with HER-2/neu-Derived Peptides Demonstrating High-Affinity Binding to Multiple Class II Alleles

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ABSTRACT

Purpose: The purpose of this study was to immunize patients with HER-2/neu-overexpressing cancer with a multipeptide vaccine comprised of four class II HER-2/neu peptides that had been identified as the most immunogenic in a previous clinical trial. Furthermore, we questioned whether MHC binding affinity could predict the in vivo immunogenicity of the HER-2/neu helper peptides.

Experimental Design: Four putative class II HER-2/neu peptides, which were found to generate detectable specific T-cell responses (stimulation index > 2) in a majority of patients in a previous study, were used to formulate a single vaccine. The multipeptide vaccine was administered intradermally with granulocyte macrophage colony-stimulating factor as an adjuvant. Ten patients with HER-2/neu overexpressing breast or lung cancer were enrolled. HER-2/neu peptide-and protein-specific T cell and antibody immune responses were measured. Competitive inhibition assays were used to analyze the class II HER-2/neu peptides for their binding affinity to 14 common HLA-DR alleles.

Results: Twenty-five percent of patients developed HER-2/neu peptide-specific T-cell immunity, and 50% developed HER-2/neu peptide-specific antibody immunity. No patient developed HER-2/neu protein-specific T cell or antibody immunity. The majority of peptides exhibited high binding affinity, in vitro, to ≥3 of the 14 DR alleles analyzed.

Conclusion: The group of peptides used in this study demonstrated high binding affinity to multiple DR alleles suggesting that in vitro binding affinity may be able to predict the in vivo immunogenicity of class II peptides. However, only a minority of patients immunized with the multipeptide vaccine developed HER-2/neu peptide-specific T cell or antibody immunity, and none developed HER-2/neu protein-specific immunity.

INTRODUCTION

Studies have now shown that tumor antigen-specific peptide-based vaccines are effective in generating immune responses to self-proteins (1, 2). Furthermore, the inclusion of well-defined MHC class II epitopes in tumor antigen-specific vaccines appears to play an important role in augmenting the immune response. A current focus of study is identifying immunogenic MHC class II epitopes of self-tumor antigens for use in peptide vaccines. In addition, methods of delivering multiple epitopes, including multipeptide vaccines, are being developed. Over the last several years the identification of MHC class II binding epitopes to common tumor antigens has been facilitated by improvements in the ability to predict MHC class II binding. More specifically, methods using quantitative binding assays, which associate in vitro high peptide-DR binding affinity with immunogenicity of class II epitopes (3), have been developed but have yet to be demonstrated in vivo.

As a result of an earlier HER-2/neu peptide vaccine study incorporating putative HER-2/neu T-helper epitopes (1), we questioned whether formulating a peptide vaccine comprised of multiple immunogenic class II peptides would result in a more robust immune response. Thus, in this study we evaluated whether active immunization with a multipeptide vaccine comprised of four HER-2/neu T-helper epitopes, which had been identified as the most immunogenic in a previous study, would generate HER-2/neu peptide- and protein-specific immunity. Furthermore, we questioned whether in vitro DR binding affinity could predict the in vivo immunogenicity of these antigenic HER-2/neu helper peptides.

MATERIALS 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 a HER-2/neu multipeptide vaccine. Ten patients with HER-2/neu overexpressing stage III (n = 5) or IV (n = 4) breast, and stage III non-small cell lung cancer (n = 1) gave informed consent, and were enrolled in the study according to institutional and federal regulations. Median age was 54 (range, 40–69) years, and median time from last chemotherapy was 10 (range, 8–16) months. Criteria for entry to the study were identical to vaccine trials described previously (1). Four peptides derived from the HER-2/neu protein sequence were formulated into a single
HER-2/neu Peptide Binding Affinity for Class II MHC.

Binding of HER-2/neu-derived peptides to HLA-DR molecules was tested using competitive inhibition assays performed as described previously (3). Briefly, purified human class II molecules, 5–500 nM, an excess of 125I-radiolabeled probe peptides, and various doses of each unlabeled peptide were coincubated for 48 h in the presence of a protease inhibitor mixture. After the incubation period, MHC-peptide complexes were separated from unbound radiolabeled peptide by one of two methods: size-exclusion gel-filtration chromatography or capture of complexes using anti-DR monoclonal antibody LB3.1. The percentage of bound radioactivity was then determined. The IC_{so} of unlabeled peptide required to inhibit the binding of the labeled peptide was determined by plotting dose versus percentage of inhibition. Under conditions where [label] < [MHC] and IC_{so} ≈ [MHC], the measured IC_{so} values are reasonable approximations of true $K_{d}$ values. Significant binding affinity was defined as 1500 nM.

Detection of Peripheral Blood T-Cell Responses. T-cell proliferation was assessed using a modified limiting dilution assay designed for detecting low frequency lymphocyte precursors based on Poisson distribution (4) and as described previously (5). Results are reported as a standard SI, defined as the mean of all 24 of the experimental wells divided by the mean of the 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 resulted in an SI > 2.0 in all of the assays reported (data not shown). Peripheral blood mononuclear cells from 30 female volunteer donors without cancer, age range 32–58, were evaluated in similar assays to establish baseline values. The mean and 3 SDs of the T-cell response in the reference population to any of the HER-2/neu antigens tested was a maximum SI of 1.98; therefore, a SI > 2 was considered evidence of an immunized response. If subjects had a SI > 2 at baseline, i.e., preexistent immunity to HER-2/neu (6), a postvaccination response was defined as positive if it was a minimum of two times baseline.

Determination of HER-2/neu Peptide Antibodies. Microwell plates (96-well; Dynex Technologies, Inc., Chantilly, VA), were coated with HER-2/neu peptides at a concentration of 20 μg/ml, diluted with carbonate buffer, and added at 50 μl/well alternating with wells coated with 50 μl/well of carbonate buffer alone. One row consisted of purified IgG (Sigma Chemical Co., St. Louis, MO) to generate a standard curve. After overnight incubation, all of the wells were blocked with 1% casein/PBS, 100 μl/well, and incubated at room temperature for 1–2 h. Plates were then washed with a 0.15% casein/1% PBS/0.05% Tween 20 wash buffer four times before experimental serum diluted in 10% FCS/PBS/1% BSA/25 μg/ml mouse IgG was added at 1:100, 1:200, 1:400, and 1:800 dilutions. Plates were incubated for 2 h at room temperature. Plates were then washed four times with casein-based wash and incubated for 45 min at room temperature after addition of 50 μl/well IgG-horseradish peroxidase conjugate (Zymed Laboratories, San Francisco, CA) diluted 1:10,000 in PBS/BSA buffer. After a final four washes with casein-based wash buffer, TMB reagent (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added 75 μl/well and color reaction read at 640 nm until the well containing the 0.16 μg/ml standard reached an absorbance of 0.3. Reactions

### Table 1

<table>
<thead>
<tr>
<th>HER-2/neu peptides</th>
<th>Number (%) of patients with SI&gt;2 to HER-2/neu peptides after vaccination</th>
<th>Number (%) of DR alleles associated with HER-2/neu MHC peptide binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>p42–56</td>
<td>7/13 (54)</td>
<td>3/14 (21)</td>
</tr>
<tr>
<td>p98–114</td>
<td>10/13 (77)</td>
<td>9/14 (64)</td>
</tr>
<tr>
<td>p328–345</td>
<td>5/13 (38)</td>
<td>0/14 (0)</td>
</tr>
<tr>
<td>p369–386</td>
<td>12/14 (86)</td>
<td>5/14 (36)</td>
</tr>
<tr>
<td>p688–703</td>
<td>5/14 (38)</td>
<td>1/14 (7)</td>
</tr>
<tr>
<td>p776–790</td>
<td>11/11 (100)</td>
<td>9/14 (64)</td>
</tr>
<tr>
<td>p972–941</td>
<td>8/11 (73)</td>
<td>0/14 (0)</td>
</tr>
<tr>
<td>p797–801</td>
<td>9/14 (64)</td>
<td>4/14 (29)</td>
</tr>
<tr>
<td>p1166–1180</td>
<td>6/11 (55)</td>
<td>1/14 (7)</td>
</tr>
</tbody>
</table>

* The number and percentage of patients who developed specific T-cell responses to 9 HER-2/neu peptides (SI>2) when those peptides were administered as a vaccine are shown (1). The number and percentage of HLA-DR alleles associated with in vitro high binding affinity of the 9 HER-2/neu peptides are also shown. Data is based on analysis of 14 HLA-DR alleles.

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The abbreviations used are: GM-CSF, granulocyte macrophage colony-stimulating factor; ECD, extracellular domain of HER-2/neu; ICD, intracellular domain of HER-2/neu; SI, stimulation index; TCR, T-cell receptor; CV, coefficient of variation.

were stopped with 75 μl/well 1 N HCL and read at 450 nm. The absorbance of each serum dilution was calculated as the absorbance of the peptide-coated wells minus the absorbance of the buffer-coated wells. Values for μg/ml were calculated from the log-log equation of the line for the standard curve on each plate. A positive sample was defined as an antibody concentration greater than the mean of a volunteer blood donor population and 3 SDs, 0.07–0.26 μg/ml (n = 108). Some patients did have pre-existent detectable antibody immunity to an immunizing peptide and were only considered to have responses if they boosted the antibody level to twice their baseline value. The specificity of the assay was 78% and sensitivity 90%. The accuracy of the HER-2/neu protein antibody assay was described with an average CV of 10%, linearity by a correlation coefficient of 0.99. On the basis of repeated measures of 20 volunteer and 20 experimental sera over a 6-month period the intra- and interassay CVs were 15% and 9%, respectively.

Statistical Methods. To determine whether the percentage of patients who developed an immune response after active immunization correlated with the number of DR alleles with significant binding for specific alleles, Pearson’s correlation coefficient was estimated. The data used for this analysis were simply the percentage of patients with a response for a particular peptide and the corresponding percentage of alleles with significant binding. No adjustment was made to account for the fact that the number of patients immunized with a particular peptide varied, as this number was relatively constant (11–14).

RESULTS

Immunodominant HER-2/neu Peptide Epitopes Determined by in Vivo Immunogenicity Also Bind MHC Class II Molecules with High Affinity. Nine peptides used in active immunization in a study reported previously (1) were analyzed for their binding affinity to 14 HLA-DR alleles (Fig. 1). Of the nine peptides used in the current study (Table 1). All 14 of the DR alleles tested were shown to have high in vitro binding affinity, defined as 1500 nM, to at least one of the nine HER-2/neu peptides originally assessed (Fig. 1). Five of 9 peptides had high binding affinity, in vitro, to ≥3 of the 14 DR alleles analyzed. p328 and p927 were not associated with significant binding of any of the DR alleles evaluated. The Affinity of in Vitro Peptide Binding Correlates with in Vivo Immunogenicity. Assessment of binding of the HER-2/neu peptides to class II MHC molecules revealed that the percentage of high binding DR alleles for a given peptide significantly correlated with the percentage of patients who...
responded to that peptide when it was administered as a vaccine, $r = 0.76$ (95% confidence interval, 0.50–1.0).

**Patients Enrolled Had MHC Class II Alleles Capable of Binding HER-2/neu Immunizing Peptides.** Three of the four immunogenic HER-2/neu peptides, p98, p369, and p776, used for vaccination had high in vitro binding affinity to $\geq 1$ of the HLA-DR alleles analyzed (Fig. 1). All 8 of the patients evaluated had $\geq 2$ DR alleles associated with high binding affinity of HER-2/neu peptides in their immunizing mix (Fig. 2).

**The Minority of Patients Immunized with the Vaccine Containing Peptides Associated with High-Affinity Binding to Multiple DR Alleles Developed HER-2/neu Peptide-Specific T-Cell Immunity, and None Developed HER-2/neu Protein-Specific Antibody Immunity.** Fig. 3 shows the pre- and postimmunization T-cell responses of the 8 patients who completed six vaccines. Two of 8 (25%) patients developed immune response to at least one of the HER-2/neu peptides, one to p369 (SI, 6.5) and one to p776 (SI, 2.4). Four patients had pre-existent immune responses to one or more peptides, 3 patients to p98 (SI, 2.0, 17.3, and 18.1), 1 patient to p369 (SI, 3.9), 3 patients to p776 (SI, 2.7, 2.8, and 2.9), and 3 patients to p927 (SI, 2.8, 6.4, and 6.7). All of the patients who had pre-existent immune responses had a decrease in SI postimmunization. None of the patients developed HER-2/neu protein-specific immunity after peptide immunization (Fig. 1). Five patients had pre-existent immune responses to either ICD or ECD. All 5 of the patients had pre-existent immune responses to ICD (range, SI, 4.2–11.6), and 3 of the 5 patients responded to ECD before immunization (range, SI, 3.2–3.5). All 5 of the patients with pre-existent immunity had a decrease in SI postimmunization.

The vaccine was very well tolerated with only five events in vivo immunogenicity also bind MHC class II molecules with high affinity in vitro. However, only a minority of patients immunized with the multipepptide vaccine developed HER-2/neu peptide-specific T cell or antibody immunity, and none developed HER-2/neu protein-specific immunity.

**DISCUSSION**

The focus of this study was to immunize patients with HER-2/neu overexpressing cancers with a multipepptide vaccine comprised of four HER-2/neu peptides that had been identified as the most immunogenic, i.e., those that generated immunity in the majority of patients immunized in a previous study (1). Additional analysis demonstrated that these four HER-2/neu peptides were associated with high affinity binding to multiple DR alleles. Thus, immunodominant HER-2/neu peptide epitopes determined by in vivo immunogenicity also bind MHC class II molecules with high affinity in vitro. However, only a minority of patients immunized with the multipepptide vaccine developed HER-2/neu peptide-specific T cell or antibody immunity, and none developed HER-2/neu protein-specific immunity.

The in vivo immunogenicity of HER-2/neu peptides could have been predicted by in vitro binding to MHC class II alleles. It is well known that MHC class I molecules recognize small peptide epitopes, 8–12 residues in size, which bind specific...
alleles (9). Binding affinity is critical to MHC class I molecules and the extent to which epitopes are able to bind determine the immunogenicity of the given epitope (10). In fact, modification of peptide motifs to increase binding results in improved immunogenicity (11). Investigators have shown that replacement of certain amino acids in the peptide sequences of carcinoembryonic antigen and gp100-derived epitopes result in enhanced MHC binding and induction of a greater number of antigen-specific cytotoxic T lymphocytes (11, 12). Several algorithms have now been developed to predict peptide-binding motifs for the most common MHC class I molecules. Unlike MHC class I, MHC class II molecules interact with larger peptides, are promiscuous, and are able to bind multiple alleles (3, 13). In vitro binding studies have suggested that high affinity binding to MHC class II molecules is associated with immunogenicity of a given peptide epitope (3). Data presented here in the present study demonstrate that binding is able to predict the immunogenicity of class II peptides. Interestingly, the peptide, p927, which was shown to be immunogenic in vivo demonstrated low affinity DR allele binding in vitro. This observation is not surprising as several studies investigating tolerance in autoimmune disease models have described epitopes displaying only negligible affinity for MHC class II molecules that occasionally become immunodominant in preference to other epitopes known to display far higher binding affinities (14). Although we made our vaccine based on the in vivo response from a previous study we could have used binding affinity to predict immunogenicity of peptides and predicted nearly the same vaccine formulation.

Unlike the parent study (1), few patients enrolled in this trial developed an immune response to HER-2/neu after active immunization with the four selected immunodominant HER-2/neu peptides. This current study used the same constructed peptides, as well as the same dose and route of administration as in the parent trial (1), so one would expect the vaccine to elicit

![Figure 3](image_url)

Fig. 3 The minority of patients immunized with the vaccine containing peptides associated with high-affinity binding to multiple DR alleles developed HER-2/neu peptidespecific T-cell immunity, and none developed HER-2/neu protein-specific T-cell immunity. Shown are pre- and postimmunization peptide- and protein-specific T-cell responses. Line graphs for ECD and ICD protein, and individual immunizing peptides are shown. Each line represents the pre- and postresponse for each patient. The dotted line defines mean and 2 standard deviations of 30 aged-matched controls.
peptide and protein specific immunity. The four peptides used were highly immunogenic in vivo, and three of the four peptides had high binding affinity for several DR alleles in vitro. Furthermore, all of the patients had HLA-DR alleles that were capable of binding one or more of the three high-binding peptides. A possible explanation for the lack of observed immune response is potential competition of the immunodominant epitopes at the level of the MHC or T cell. MHC-restricted epitopes have to compete with other peptides presumably at different levels, and several factors such as enzymatic stability, uptake, intracellular transport, number of peptide molecules, flanking sequences, MHC binding, and T-cell repertoire may influence the selection of immunogenic peptides (15). Competition at the level of the T cell may also occur, and studies have shown that T cells compete directly with each other for antigen suggesting that epitope dominance may be the result of competitive interactions between antigen-bearing antigen-presenting cells and T cells (16). Of note, however, studies in animal models using vaccines comprised of multiple class I epitopes have not supported lack of immunogenicity due to competition (17). Recent investigations using multiepitope polypeptide vaccines comprised of high binding epitopes (9 cytotoxic T lymphocytes and pan-DR epitope epitopes) have been shown to induce both CD8+ IFN-γ and T-helper lymphocyte immune response (17).

Another potential reason for the lack of immune response after active immunization is theoretical immunosuppression in advanced-stage disease cancer patients. Although the majority of patients in this study had advanced-stage disease they were treated to a minimal or nondetectable disease state before enrolling in study. Furthermore, the patients in this study were similar to the cohort of patients in the previous study (1) in regards to age, disease burden, and time from last chemotherapy. Lymphocyte phytohemagglutinin responses in this popula-

Fig. 4 The minority of patients immunized with the vaccine containing peptides associated with high-affinity binding to multiple DR alleles developed HER-2/neu peptide-specific antibody immunity, and none developed HER-2/neu protein-specific antibody immunity. Shown are pre- and postimmunization peptide- and protein-specific antibody responses. Line graphs for HER-2/neu protein and individual immunizing peptides are shown. Each line represents the pre- and postresponse for each patient. The dotted lines define mean and standard deviation of controls for HER-2/neu peptide and protein antibody assays.
tion did not differ significantly from normal donors (data not shown). Previous peptide vaccine studies by our group have demonstrated the ability to induce significant immune responses in heavily treated patients with minimal disease burden (1, 2).

Our goal was to determine the immunogenicity of a multipeptide vaccine. Although predicted to be immunogenic by a previous study, the selected immunodominant peptides did not elicit immunity in vivo when formulated into a single vaccine. However, in vitro binding studies of the selected peptides did demonstrate high binding affinity, which correlated with the development of immune response seen in the previous study. To our knowledge, this is one of the first studies to demonstrate that in vitro MHC binding affinity can predict the in vivo immunogenicity of MHC class II peptides. This correlation has significant implications in identifying immunogenic peptides when designing multipeptide vaccines. However, the combination of immunodominant epitopes in a single vaccine may inhibit immunogenicity.

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