Abstract

Purpose: Over the past two decades, there has been significant interest in targeting HER-2/neu in immune-based approaches for the treatment of HER-2/neu+ cancers. For example, peptide vaccination using a CD8 T cell–activating HER-2/neu epitope (amino acids 369-377) is an approach that is being considered in advanced phase clinical trials. Studies have suggested that the persistence of HER-2/neu–specific CD8 T cells could be improved by incorporating human leukocyte antigen (HLA) class II epitopes in the vaccine. Our goal in this study was to identify broad coverage HLA-DR epitopes of HER-2/neu, an antigen that is highly expressed in a variety of carcinomas.

Experimental Design: A combination of algorithms and HLA-DR–binding assays was used to identify HLA-DR epitopes of HER-2/neu antigen. Evidence of preexistent immunity in cancer patients against the identified epitopes was determined using IFN-γ enzyme-linked immunosorbent spot (ELIspot) assay.

Results: Eighty-four HLA-DR epitopes of HER-2/neu were predicted, 15 of which had high binding affinity for ≥11 common HLA-DR molecules. A degenerate pool of four HLA-DR–restricted 15-amino acid epitopes (p59, p88, p422, and p885) was identified, against which >58% of breast and ovarian cancer patients had preexistent T-cell immunity. All four epitopes are naturally processed by antigen-presenting cells. Hardy-Weinberg analysis showed that the pool is useful in ∼84% of population. Lastly, in this degenerate pool, we identified a novel in vivo immunodominant HLA-DR epitope, HER-2/neu88-102 (p88).

Conclusion: The broad coverage and natural immunity to this epitope pool suggests potential usefulness in HER-2/neu–targeting, immune-based therapies such as vaccines. Clin Cancer Res; 16(3); 825–34.

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p369-384, which encompasses E75, elicits E75-specific T-cell immunity that persists over 1 year (4, 5). The results indicate that, for HLA class I peptide vaccines to induce effective long-lasting immune responses, it is important to have CD4 T-cell help (11).

CD4 T cells are well known to have a fundamental role in tumor antigen-specific immunity (12). They enhance tumor antigen-specific immune responses (a) by activating CTLs through cytokines or by directly interacting with costimulatory molecules such as CD27, CD134, and major histocompatibility complex class II antigen expressed on the surface of CTLs; (b) by mediating cell death of tumor cells through apoptotic mechanisms such as Fas/FasL pathway and granzyme-perforin–dependent pathway; and (c) by activating effectors of the innate immune system such as macrophages and eosinophils (11). Previous studies showed that CD4 T cells, when used along with CD8 T cells in adoptive T-cell immunotherapy, induced cancer regression in ∼50% of melanoma patients (13). These important properties of CD4 T cells have led to considerable interest in identifying CD4 T cell–defined tumor antigen epitopes with the aim of using them as vaccines. CD4 T-cell epitopes of many different tumor antigens, such as carcinoembryonic antigen, folate receptor α, HER-2/neu, insulin, MART1/Melan-A, NY-ESO-1, p53, tyrosinase, and insulin-like growth factor–binding protein 2, have been identified in recent years (14–20).

Although several CD4 T-cell epitopes of different tumor antigens have been reported, their use as cancer vaccines is hindered by the broad polymorphism at the major histocompatibility complex class II locus. In addition to this, major histocompatibility complex class II gene frequency is usually low, typically <15% in any population compared with major histocompatibility complex class I gene frequency, which is usually high (e.g., 40-50% in the case of HLA-A2). These issues need to be considered when designing a CD4 T-cell epitope vaccine to cover a large percentage of the population. Several HLA-DR variants overlap in their binding characteristics to different epitopes. Thus, identification of

### Table 1. Binding affinities of HER-2/neu peptides to purified HLA-DR

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Peptide name</th>
<th>Position*</th>
<th>IC₅₀ nmol/L to purified HLA-DRB1*0101</th>
<th>IC₅₀ nmol/L to purified HLA-DRB1*0301</th>
<th>IC₅₀ nmol/L to purified HLA-DRB1*0401</th>
<th>IC₅₀ nmol/L to purified HLA-DRB1*0404</th>
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**NOTE:** Peptides that constitute degenerate pool are in bold. Abbreviation: ND, not determined.

*Position of N-terminal amino acid.
degenerate epitopes that bind to several HLA-DR variants should increase the efficacy and scope of CD4 T-cell epitope-based immunotherapies. In our previous studies, we developed an approach that permits identification of broad coverage pools of degenerate epitopes of tumor antigens such as insulin-like growth factor–binding protein 2, carinoembryonic antigen, and folate receptor α (15, 20, 21).

In the current study, a panel of 84 HLA-DR–binding epitopes of HER-2/neu was predicted using a computer-based predictive algorithm called predicted half maximal inhibitory concentration (IC50; ref. 20). From this candidate panel, a pool consisting of four HLA-DR epitopes of HER-2/neu was identified. HLA-DR epitopes that constitute this pool are immunogenic, naturally processed, and cover ∼84% of diverse population, including Caucasians, African Americans, and Asians (Hardy-Weinberg equilibrium analysis). A novel HER-2/neu HLA-DR–restricted epitope, p88, was identified in this degenerate pool as an in vivo immunodominant epitope.

Materials and Methods

Reagents. The peptides used in this study were synthesized at Mayo Clinic Proteomics Core Facility or at Epimmune, Inc. Purity of the peptides was determined by reversed-phase high-performance liquid chromatography and amino acid analysis, sequencing, and/or mass spectrometry. Lyophilized peptides were diluted in DMSO and PBS. Recombinant human ErbB2 protein, HER-2/neu cytoplasmic domain (676-1,255 amino acids), and partial ErbB2 recombinant protein (22-122 amino acids) were obtained from Invitrogen and Novus Biologicals, respectively. HER-2/neu entire extracellular domain protein (1-627 amino acids) was generously provided by Raphael Clynes (Columbia University, New York). Cytomegalovirus, Epstein-Barr virus (EBV), and influenza virus (CEF) viral peptide pool was obtained from NIH AIDS Research and Reference Reagent Program.

Patients and donors. This study was approved by Institutional Review Boards at Mayo Clinic and University of Washington, Seattle, WA. Blood samples were obtained from 18 female healthy donors and 38 patients (9 breast and 29 ovarian) at Mayo Clinic. Samples of 10 breast cancer patients were obtained from the University of Washington, Seattle, WA. Samples from both institutions were processed and stored in a similar fashion. All the patients were disease free at the time of sample collection. Formalin-fixed, paraffin-embedded tissue samples were obtained from the patients at the time of initial surgical procedure (6 breast tumor and 20 ovarian tumor). The mean ages of healthy donors and patients were 42 ± 11 and 55 ± 2 y, respectively (P < 0.0001). Tumor grade and stage information were available for patients, as described previously (20). There were no differences in T-cell responses between patients and healthy donors against nonspecific stimulus, phorbol 12-myristate 13-acetate/ionomycin, and CEF viral antigens (20).

Preparation of peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) were isolated from blood by density-gradient centrifugation, as described previously (20). Cells were cryopreserved in liquid nitrogen in freezing medium (RPMI 1640 with fetal bovine serum and DMSO) at a cell density of 25 to 50 × 10⁶ cells/mL.

Epitope prediction. Predicted IC50, a modified linear coefficient and matrix-based method, was used for predicting

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<th>DRB1*0802</th>
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<td>1,408</td>
<td>901</td>
<td>227</td>
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HLA-DR–binding capacity of peptides (22, 23). Lower predicted IC_{50} values indicate higher binding affinity of peptides to a HLA class II molecule.

**HLA-DR purification.** HLA-DR molecules were chosen for this study to allow balanced population coverage (24). HLA-DR molecules were purified from EBV-transformed homozygous cell lines or from transfected fibroblast cell lines (23, 25). Cell lysates were passed through LB3.1 monoclonal antibody (mAb) columns, and HLA class II molecules were eluted at high pH (pH 11.5), followed by pH reduction to 8.0. Eluates were concentrated by centrifugation.

**HLA-DR–binding assays.** The binding affinity of peptides to different HLA-DR molecules was determined by their ability to inhibit the binding of high-affinity radiolabeled probe peptides to specific HLA-DR molecules using a solid-phase capture RIA (26). Briefly, purified HLA-DR molecules and radiolabeled peptides were incubated in the presence of the inhibitor peptide in a reaction vessel for 2 d either at room temperature or at 37°C in the presence of protease inhibitors. After incubation, the percentage of HLA-DR bound radioactivity was determined by capturing HLA-DR/peptide complexes on Optiplates (Packard Instruments) coated with the mAb LB3.1 and determining bound counts per minute followed by affinity calculations. As in previous studies, peptides with affinities for specific HLA-DR molecules of 1,000 nmol/L or better were defined as high-affinity binders.

**Enzyme-linked immunosorbent spot.** A 10-d enzyme-linked immunosorbent spot (ELIspot) assay was used to determine reactivity of low-frequency T cells to HER-2/neu peptides (Table 1) and was done in groups of two (two healthy donors, one healthy/one cancer patient, or two cancer patients), as previously described (20). A patient was considered as a responder to a specific peptide if the mean T-cell frequency was higher than mean T-cell frequency plus 2 SDs of control population (20). A peptide was considered naturally immunogenic if >10% of the patients showed significantly elevated immunity to that peptide relative to the controls.

**Generation of HER-2/neu–specific CD4+ T cells.** Dendritic cells were generated from PBMCs, as described previously (20). Briefly, PBMCs were cultured in modified RPMI medium in six-well tissue culture plates (6 × 10^6 cells per well) for 2 h at 37°C. Nonadherent cells were removed, and adherent cells (monocytes) were cultured further in the presence of hGM-CSF (800 U/mL) and hIL-4 (1,000 U/mL) at 37°C. On day 5, CpG (1 μg/mL) was added to each well. On day 6, dendritic cells were pulsed with peptide (10 μg/mL) for 4 h in the presence of T cells.
of B7-dendritic cell cross-linking antibody (a gift from Dr. Larry Pease, Mayo Clinic). Pure CD4 T cells isolated from PBMCs by isolation kit (Miltenyi Biotec, Inc.) were added to peptide pulsed dendritic cells, and the cultures were incubated at 37°C with periodic interleukin 2 (10 U/mL) and interleukin 12 (10 ng/mL) addition. On day 15, cells were assayed for reactivity with the HER-2/neu antigen (HER-2/neu peptides and HER-2/neu protein) and irrelevant antigens by ELispot. For this assay, in vitro stimulated CD4 T cells (1 × 10^5 cells per well) and autologous irradiated PBMCs (1 × 10^5 cells per well) were added at 1:1 ratio in each well in 96-well nitrocellulose (NC)-plates coated with anti-human IFN-γ antibody and incubated at 37°C at 5% CO2 for 20 to 24 h in the presence of different stimuli. Stimuli were respective HER-2/neu peptide (10 μg/mL) and either the HER-2/neu cytoplasmic domain (676-1,255 amino acids), a partial HER-2/neu extracellular domain recombinant protein (22-122 amino acids), or HER-2/neu entire extracellular domain protein (1-627 amino acids; 1 μg/mL). For the irrelevant peptide, human collagen II peptide, HII.71 (PPGLTGPAGEPGRQGSPGAD; 10 μg/mL), was used, and for irrelevant protein, ovalbumin was used. Results are expressed as antigen-specific CD4 T cells per 1 × 10^5 CD4 T cells. To determine HLA class II restriction of peptide-specific CD4 T cells, HLA-DR and HLA-DP, HLA-DQ, and HLA-DR–specific antibodies (BD Biosciences; 10 μg/mL) were used in ELispot assay.

**Immunohistochemistry.** Immunohistochemical staining was done using Food and Drug Administration–approved Hercep Test kit (DAKO K5207). Briefly, formalin-fixed, paraffin-embedded tissue sections were deparaffinized, and antigen retrieval was carried out using citrate retrieval buffer. Slides were treated with peroxidase-blocking reagent, followed by incubation with prediluted rabbit polyclonal primary antibody for 30 min at room temperature. Visualization reagent was applied for 30 min at room temperature, followed by a 10-min incubation at room temperature with dianamobenzidine. Sections were counterstained with hematoxylin. Staining intensity was graded on a 0 to 3 scale: 0 and 1 grades were considered as low expression, and 2 and 3 grades were considered as high expression. Staining was done in a blinded fashion.

**Statistical analysis.** Statistical analyses were done using GraphPad Instat Software or GraphPad prism software. Two-tailed Mann-Whitney tests or Student’s t tests were used to analyze the data unless otherwise stated. P < 0.05 was considered as significant.

**Results**

HER-2/neu peptides have high binding affinity to HLA-DR molecules. Eighty-four HER-2/neu HLA-DR epitopes were predicted using predicted IC_{50}. All 84 peptides were
synthesized and tested for binding to HLA-DR molecules, as described in the Materials and Methods. Fifteen of the 84 peptides were selected based on high-affinity degenerate (≤1,000 nmol/L) binding to at least 11 of 15 HLA-DR molecules (Table 1). These peptides were resynthesized at high purity and tested for reactivity in peripheral blood samples from normal healthy donors and breast or ovarian cancer patients using IFN-γ ELISPOT assays. As predicted, these peptides bound with high affinity to DRB1*0101 molecule (Table 1).

**Breast and ovarian cancer patients have elevated HER-2/neu epitope pool-specific T-cell immunity.** PBMCs from breast and ovarian cancer patients were used in ELISPOT assays to determine the frequency of T cells against the 15 peptides selected on the basis of binding assay results. A pool of four peptides (p59, p88, p422, and p885) was identified, against which >10% of patients had elevated T-cell responses. Mean T-cell frequencies of responders were in the range of 314 ± 64 to 635 ± 172 (Fig. 1), higher than T-cell frequencies detected among healthy donors. There were no significant differences (P > 0.05) in epitope-specific T-cell responses between breast and ovarian cancer patients. In addition, cumulative T-cell frequency against the pool observed in patients was significantly higher than in controls (P = 0.006; Fig. 2A). Fifty-eight percent of the patients and 22% of the controls responded to the pool (P = 0.009; Fig. 2B). Although our results show that only 58% of patients responded to peptide pool challenge, Hardy-Weinberg analysis suggests that this pool would be useful in 84% of patients (Table 2).

**T-cell responses against the peptide pool are HER-2/neu expression dependent.** Tumor tissues were obtained from breast and ovarian cancer patients, and immunohistochemical staining was done to determine whether T-cell responses observed in patients correlated with HER-2/neu expression in tissue samples. In our previous studies, we reported that breast cancer patients treated with trastuzumab and chemotherapy have increased HER-2/neu–specific CD4 T-cell responses (27). Because the breast cancer patients selected for this study were under active treatment at some point before the collection of blood samples, we have excluded the T-cell response results from these patients in correlation analysis to avoid the bias. Based on the staining intensity, patient samples were divided into high and low expression categories (Fig. 2C). As shown in Fig. 2D, patients with high levels of HER-2/neu expression (n = 6) in their tumors had elevated T-cell responses against the pool compared with T-cell responses observed in patients with low levels of HER-2/neu expression (n = 16; P = 0.04).

**Peptides that constitute the epitope pool are naturally processed.** To determine whether HLA-DR epitopes of the pool are naturally processed, peptide-specific CD4 T cells were generated as described in Materials and Methods. These CD4 T cells were tested for their reactivity against HER-2/neu protein and individual HER-2/neu HLA class II peptides. As shown in Fig. 3, peptide-specific CD4 T cells responded to HER-2/neu protein and peptides. There were no discernable differences between the reactivity of CD4 T cells against peptides and HER-2/neu protein (P > 0.05). These results indicate that the four epitopes that constitute the pool are naturally processed and presented by antigen-presenting cells to CD4 T cells. These experiments were repeated thrice using CD4 T cells isolated from three different donors.

**p88 is an in vivo immunodominant epitope of HER-2/neu.** We also correlated HER-2/neu expression in tumors with T-cell responses against individual peptides constituting the pool. As shown in Fig. 4A, patients with HER-2/neu**19** tumors had elevated T-cell responses against p88 peptide. This response is significantly greater than p88–specific T-cell responses seen in patients with HER-2/neu**19**
tumors. However, the differences in p59-, p422-, and p885-specific T-cell responses among patients with HER-2/neu\textsuperscript{hi} tumors and HER-2/neu\textsuperscript{lo} tumors were not statistically significant (data not shown). Patients with HER-2/ neu\textsuperscript{hi} tumors had significantly higher p88-specific T-cell responses compared with the T-cell responses against the other epitopes (p59, p422, and p885) comprising the pool (Fig. 4B). No significant differences ($P > 0.05$) in T-cell responses were observed among patients with immunohistochemistry (IHC)0, IHC1+, and IHC2+ tumors. These results suggest that T-cell responses observed in the patients correlate with HER-2/neu expression in tumors of the patients with the immunodominant T-cell responses being p88 specific. Thus, we conclude that p88 is an \textit{in vivo} immunodominant epitope of this pool. To verify that the p88 epitope was HLA-DR restricted, p88-specific T cells were generated and tested for reactivity against p88 peptide in the presence or absence of HLA-DR antigen-specific mAb. As shown in Fig. 4C, epitope-specific responses were completely blocked by the inclusion of two different antibodies recognizing HLA-DR but not of an isotype control antibody.

**Discussion**

HER-2/neu-based peptide vaccines have been used for the past several years to treat different types of cancers. Much focus has been on the development of HLA class I peptide vaccines, which have the ability to elicit tumor antigen-specific CTLs. Recently, there has been considerable interest in incorporating peptides that also activate CD4 T cells because they have a prominent role in prolonging effector CD8 T-cell responses and maintaining immunologic memory. Thus, identification of novel HLA class II epitopes of HER-2/neu may facilitate the development of effective multiepitope vaccination strategies against different cancers using HER-2/neu-based peptide vaccines. The genetic diversity at the HLA class II locus poses a significant problem, however, requiring the identification of degenerate epitopes that can be used in broader patient populations. In this study, we identified a pool of four naturally processed degenerate HLA-DR epitopes of HER-2/neu antigen, against which breast and ovarian cancer patients had elevated T-cell immunity. This pool is predicted to be recognized by up to ∼84% of the population and each peptide covers a minimum of 12 of 15 major HLA-DR alleles. Characterization of the immunogenicity of this pool led to the identification of a novel \textit{in vivo} immunodominant epitope, p88. Although other CD4 T-cell epitope pools for HER-2/neu have been established, the pool in the current report seems to be superior in terms of coverage. For example, using an older algorithm (TSites) in the 1990s, Disis et al. (28) developed three different HER-2/neu pools, an intracellular domain pool (p776,
p927, and p1166), an extracellular domain pool (p42, p98, and p328), and an HLA-A2 pool (p369, p688, and p971). Together, these nine peptides cover 11 of 15 HLA-DRs examined with only a mean coverage of two epitopes per allele. In contrast, the pool of four epitopes identified in the current study covers 14 of 15 alleles examined, with a mean coverage of four epitopes per allele.

Some self-proteins or tumor antigens are overexpressed in tumor cells compared with their expression in normal cells. These tumor antigens can be immunogenic if they are presented along with costimulatory molecules and other danger signals in the tumor microenvironment or regional lymph nodes. Thus, it is possible to observe correlations between expression of tumor antigens in tumor cells and endogenous immune responses against these tumor antigens in cancer patients. Goodell et al. (29) reported that overexpression of HER-2/neu protein in tumors correlates with endogenous T-cell responses observed in breast cancer patients. By immunohistochemical analyses, we determined that patients with HER-2/neu hi tumors had elevated T-cell responses against the pool compared with patients with HER-2/neu lo tumors, and most T-cell responses observed in patients with HER-2/neu hi tumors were p88 specific. In addition, it was observed that the proportion of patients responding to p88 epitope (23%) was higher than that of patients responding to other epitopes [p59 (16%), p422 (16%), or p885 (15%)] in the pool. Based on their findings Perez et al. (30) suggested that HER-2/neu protein might have lysosomal targeting sequences, which would allow it to be processed in endogenous major histocompatibility complex class II pathway. Our data indicate that all the epitopes in the pool, including p88, are derived from natural processing of HER-2/neu protein, suggesting that the p88 sequence in the HER-2/neu protein could be targeted by lysosomes and ultimately presented by major histocompatibility complex class II molecules after being processed in endogenous major histocompatibility complex class II pathway. Moreover, inhibition experiments confirm HLA-DR restriction of p88 peptide. Thus, we speculate that with the increase in the expression of HER-2/neu in tumor cells, p88 epitope is presented on the surface of tumor cells at a higher rate compared with other epitopes in the pool. Taking into consideration all the above facts, we concluded that p88 is an in vivo immunodominant epitope. Because immunodominance of the epitope is influenced by several factors such as HLA binding, TCR binding, stability, and abundance of the peptide (20), in the future, it will be necessary to design studies including this p88 peptide as a component in HER-2/neu cancer vaccines to address the significance of our finding.

In addition to incorporation with HLA class I epitopes, there are other potential applications of the degenerate epitope pool described in the current study, such as including it with trastuzumab therapy. Trastuzumab (Herceptin) is a humanized mAb against HER-2/neu protein, and it is a drug of choice for the treatment of HER-2/
neu+ breast cancer patients with metastatic disease (31). It has been suggested that trastuzumab acts on cells by enhancing the internalization of HER-2/neu protein and ultimately resulting in increased surface expression of HLA class I peptides on the surface of tumor cells, thereby increasing their susceptibility to the lytic effects of CTLs (32, 33). Taylor et al. (27) also showed that trastuzumab treatment enhanced CD4 T-cell and antibody responses in breast cancer patients, immunity that correlated well with clinical responses to therapy. Given the importance of CD4 T cells in CTL and antibody responses (11), the use of HER-2/neu CD4 T-cell epitopes along with trastuzumab may boost clinical efficacy. Given the degeneracy of these epitopes, we speculate that the vaccine regimen should be useful in large population. In addition, the identified pool of epitopes may be useful as biomarkers to predict patient’s responsiveness to trastuzumab-based therapy.

Although we reported a pool of only four HLA-DR epitopes of HER-2/neu antigen that might be useful in future studies as a vaccine cancer regimen, the potential use of the other HLA-DR epitopes reported in this study should not be overlooked. For example, our HLA-DR binding affinity data indicate that epitopes, such as p83, p432, and p783, bind at least 13 of 15 HLA-DR variants, but elevated peptide-specific T-cell responses were not observed in the current group of patients. Several factors, such as lack of natural processing of these epitopes despite their high binding affinity to several HLA-DR variants, natural deleterious mechanism of T cells responding to these epitopes, and inhibition of T cells responding to these epitopes by regulatory T cells, could account for the lack of responsiveness by the patients (15, 20). Future studies addressing these issues could determine whether these epitopes also constitute an immunogenic degenerate HLA-DR epitope pool of HER-2/neu antigen. In fact, these epitopes (p432 and p783) encompass HLA class I epitopes p435 and p785, which were reported as potential CTL epitopes in previous studies (7, 34). In addition, we predicted that 84% of patients could respond to the degenerate pool, but we observed only 58% of patients had elevated immunity against the pool. Several factors, such as immunodominance, immunosuppression, and, HER-2/neu expression in the patients selected for this study, can be attributed to the differences in predicted and observed responses (15).

In summary, in this study, we identified a pool of degenerate HLA-DR epitopes of HER-2/neu antigen, against which breast and ovarian cancer patients have endogenous immunity and which consists of a novel in vivo immunodominant HLA class II epitope, p88. We predict that, in future studies, this pool would be useful in the development of successful peptide-based immunotherapeutic strategies against HER-2/neu+ cancer patients and also would serve as an effective tool in determining immune responses of cancer patients treated with HER-2/neu-based vaccine regimen.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

11. Knutson KL, Disis ML. Tumor antigen-specific T helper cells in
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