Proliferative and Cytokine Responses to Class II HER-2/neu-associated Peptides in Breast Cancer Patients


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ABSTRACT

Previous studies have characterized the reactivity of CD8+ CTLs with ovarian and breast cancer. There is little information about the antigens and epitopes recognized by CD4+ T cells in these patients. In this study, we analyzed the ability of T cells from peripheral blood mononuclear cells of breast cancer patients to recognize HER-2/neu (HER-2) peptides. We found that 13 of 18 patients responded by proliferation to at least one of the HER-2 peptides tested. Of these peptides, one designated G89 (HER-2: 777-789) was recognized by T cells from 10 patients. Seven of nine responding patients were HLA-DR4+, suggesting that this peptide is recognized preferentially in association with HLA-DR4. Analysis of the specificity and restriction of the cytokine responses to G89 by G89-stimulated T cells revealed that these cells secreted significantly higher levels of IFN-γ than interleukin 4 and interleukin 10, suggesting priming for a Th0-Th1 helper 1 response. The same pattern of cytokine responses was observed to the intracellular domain of HER-2 protein, suggesting that G89-stimulated T cells recognized epitopes of the HER-2 protein in association with HLA-DR4. Because HLA-DR4 is present in 25% of humans, characterization of MHC class II-restricted epitopes inducing Th0-Th1 helper 1 responses may provide a basis for the development of multivalent HER-2-based vaccines against breast and ovarian cancer.

INTRODUCTION

Studies in animal models have demonstrated a significant role for T lymphocytes in antitumor immunity and have shown that CD8+ and CD4+ cells can mediate tumor rejection (1, 2). In recent years, significant emphasis has been placed on identifying epitopes recognized by tumor-reactive CD8+ CTLs. A remarkable feature of these Ags is that they are nonmutated self proteins (3). This raises the possibility that CD4+ cells recognizing epitopes on the same self proteins in the context of MHC I and MHC II may also be present in cancer patients (4). CD4+ cells may either express direct killing or play a regulatory role in the differentiation of other CD4+ cells and of tumor-reactive CTLs (5).

The known repertoire of tumor Ag recognized by CD4+ cells is limited. There is little information on the restriction elements operating in each Ag system and the nature of responses (Th1/Th2) induced by self peptides activating CD4+ cells. Topalian et al. (6, 7) have identified HLA-DR4.1 (HLA-DRB1*0401)-restricted tyrosinase peptides that stimulated Th1 cytokine secretion by CD4+ melanoma TILs. This ability was shown to be dependent on the binding affinity of the peptide to HLA-DR (7). Yoshino et al. (8) have shown that CD4+ TILs secrete Th1 cytokines when presented with heat shock proteins associated with HLA-DR, suggesting that they may recognize peptides complexed to such proteins (8). CD4+ CTLs were shown to recognize a shared HLA-DR15 melanoma-associated Ag (9). MHC class II-restricted Th1 cytokine secretion by long-term cultured CD4+ TILs has also been reported in breast cancer patients, suggesting a HLA-DR4-associated response (10). Finally, autologous tumor-specific CD4+ CTLs have also been demonstrated in sarcoma restricted by HLA-DR4 and HLA-DR15 (11).

These studies have shown that in vitro cultured CD4+ cells of TILs can recognize class II-associated Ag. There is little information on the ability of class II-associated peptides from these Ags to induce and restimulate a response in healthy donors and patients with cancer. Proliferative responses of PBMCs reflecting responses by T cells to mutated Ras protein and peptides carrying the same mutation have also been detected in pancreatic and colon cancer patients vaccinated with the corresponding peptide (12, 13). In contrast, responses to w.t. or
mutated Ras have not been found in healthy donors (12). However, the immunity directed against mutated tumor proteins may be targeted to w.t. epitopes (14, 15). Breast cancer patients developed anti-p53 antibodies and T cells that proliferated in vitro in response to w.t. p53 only when mutated p53 accumulated in their tumors (15), suggesting that the enhanced presentation of w.t. p53 was leading to a Th2 response. Similarly, a breast cancer patient with HER-2 overexpression (HER-2\textsuperscript{234}) and anti-HER-2 antibodies developed T cells that proliferated in response to both HER-2 protein and short HER-2 peptides (16).

These results suggest an in vivo priming by enhanced presentation of self peptides due to HER-2 protein overexpression. There is little information on the ability of CD4+ cells from healthy donors and from cancer patients who do not overexpress HER-2 (HER-2\textsuperscript{234}) to respond to HER-2 peptides. This is important because identification of CD4+ cells reacting with self peptides may allow not only identification of “protective” tumor Ag, but allow optimization of design of tumor vaccines, by incorporating a “self helper” peptide(s) that can amplify and spread a Th1 response when the disease progresses.

We recently found that healthy donors responded with higher frequency than ovarian cancer patients to a number of HER-2 peptides. We hypothesized that CD4+ cells recognizing HER-2 are not deleted from the immune repertoire of healthy individuals (17). Because the patients in that study were not HLA typed and had advanced disease, we decided to investigate the ability of HER-2 peptides to induce proliferative responses in healthy patients with primary breast cancer of defined MHC class II type. We focused on two HER-2 peptides that induced the most frequent responses in our previous study. We found that peptide G89 (HER-2, 777–789) induced responses with higher frequency (10 of 18, 56%) in this group and a number of HER-2 peptides tested. There was no difference in the pattern of cytokine responses between one patient who overexpressed HER-2 (HER-2\textsuperscript{234}) and one healthy donor who did not overexpress HER-2 (HER-2\textsuperscript{234}), suggesting that the ability of patients with localized breast cancer to respond to G89 it is not affected by HER-2 overexpression.

MATERIALS AND METHODS

Subjects. PBMCs were obtained from 18 breast cancer patients and 6 healthy volunteers (three DR4+ and three DR4–). All patients, with one exception, were clinically free of tumor at the time of study. Of the healthy volunteers, three were HLA-DR4+, the others were HLA-DR4– (i.e., MHC II phenotype was: donor 4, DR7, 11, DQ2, 6; donor 5, DR13, 14; donor 6, DR11, 15, DQ6, 7). Eleven patients had pathology stage I disease, 5 had stage II, and 1 had stage III. One patient (patient 16) had no primary tumor yet was classified as having breast cancer because of the presence of a positive lymph node. All except one patient had undergone surgery and were free of disease at the time of study. One patient had recurrent disease. All patients except one had 0–3 positive lymph nodes. Tumor from only one patient had Black’s nuclear grade III (advanced pathological characteristics); the remainder were grade I or II.

HLA Class II Molecular Oligotyping. Genomic DNA extracted from PBMCs as described (18–22) served as the substrate for amplification of a polymorphic locus-specific fragment of the HLA class II gene by PCR. For the -DQB\textsubscript{1} and -DRB loci, the flanking primers used were as follows: DRB-AMP-A, 5’CCCCACAGCACCGGTTCTG; DRB-AMP-B, 5’CGCTGCACCTGTTCTCCTG; -DQB-AMP-A, 5’CATGGTGTGACTCTCACAACG; and -DRB-AMP-B, 5’CTCTGATGTTTCTGGCACAC.

Because of the large number of HLA-DRB alleles and the numerous shared sequences between different alleles, HLA-DRB typing was carried out in a stepwise manner. First, group-specific HLA-DR typing was performed using the primers DRB-AMP-A and DRB-AMP-B. Oligonucleotide typing of this PCR-amplified DNA allowed discrimination of HLA-DR\textsubscript{1}, DR\textsubscript{2}, DR\textsubscript{3/6}, DR\textsubscript{4}, DR\textsubscript{5} (DR\textsubscript{w11}), DR\textsubscript{7}, DR\textsubscript{8/12}, DR\textsubscript{9}, DR\textsubscript{10}, DR\textsubscript{52a}, DR\textsubscript{52b/c}, and DR\textsubscript{w53}. Because there are numerous variants of HLA-DR\textsubscript{1}, DR\textsubscript{2}, DR\textsubscript{4}, DR\textsubscript{5} (DR\textsubscript{w11}), DR\textsubscript{6}, DR\textsubscript{8/12}, and DR\textsubscript{52b/c}, further discrimination of these subtypes required a second PCR using group-specific primers plus DRB-AMP-B. They include DRB-AMP-1 for the HLA-DR\textsubscript{1} group, DRB1-AMP-2 or DRB5-AMP-2 for the HLA-DR\textsubscript{2} group, DRB-AMP-3 for the HLA-DR\textsubscript{3}, DR\textsubscript{5}, DR\textsubscript{6}, DR\textsubscript{8}, DR\textsubscript{12} group, DRB-AMP-4 for the HLA-DR\textsubscript{4} group, and DRB-AMP-52 for the HLA-DR\textsubscript{53} genes of the HLA-DR52 group. The sequences of the primers were as follows: DRB-AMP-1, 5’TCTCTTGCGCCATCTATGTT; DRB1-AMP-2, 5’TCTCTGTGCACTTAGAG; DRB5-AMP-2, 5’CATGGTGTGACTCTCACAGG; DRB-AMP-4, 5’CTCTGATGTTTCTGGCACAC.

For HLA-DRw52-associated -DQB\textsubscript{1} genes (HLA-DR\textsubscript{3-5}, DR\textsubscript{6}, DR\textsubscript{8}, and DR\textsubscript{12}), the sequences of the primers were as follows: DRB-AMP-3 (5’CAGCTTCTCTGAGCAGCAGG); DRB-AMP-52 (5’CCCGACCTTCTGGCAGG). PCR products separated by electrophoresis were blotted to Hybond N+ membranes (Amer sham Pharmacia Biotech, Arlington Heights, IL) hybridized with [\gamma\textsuperscript{32}P]ATP-labeled allele-sequence-specific oligonucleotide probes. HLA-DQB\textsubscript{1} allelic variants were determined by hybridization with probes corresponding to variable sequences around positions 23, 26, 37, 45, 49, 57, and 70 of the HLA-DQB\textsubscript{1} outermost domain. “Broad” HLA-DR groups [HLA-DR\textsubscript{1}, DR\textsubscript{2}, DR\textsubscript{3/6}, DR\textsubscript{4}, DR\textsubscript{5} (11), DR\textsubscript{12}, DR\textsubscript{7}, DR\textsubscript{8}, DR\textsubscript{9}, DR\textsubscript{10}, DRB3*0101, DRB3*0201, DRB4*0101 (DR\textsubscript{53})] were determined by hybridization with oligonucleotide probes corresponding to variable sequences around positions 10, 28, and 37 of the HLA-DRB1 outermost domain. Subtypes of HLA-DR\textsubscript{1}, DR\textsubscript{2}, DR\textsubscript{3/6/8/12}, DR\textsubscript{4}, and DR\textsubscript{w52} were determined by hybridization of the respective group-amplified DNA to oligonucleotides corresponding to variable sequences around positions 28, 37, 57, 70, and 86 of the HLA-DRB1 outermost domain.

HER-2 Peptide Selection. Peptides tested were selected if they contained the T-cell sites in HER-2 predicted by the computer program AN.T.FIND.M, the general binding motif for human class MHC II Ag, and the anchors for a number of MHC class II Ags (HLA-DR\textsubscript{1}, DR\textsubscript{3}, DR\textsubscript{4}, DR\textsubscript{11}, and -DQ7; Refs. 23–28). The sum of whose allelic frequencies covers 75–100% of Americans. The general peptide binding motif for various human MHC class II molecules consists of a P1 anchor, i.e., an aromatic or large aliphatic residue in the first 3–5 amino acids.
greater than 95% combination of using a pET vector with an amino-terminal His Escherichia coli (data not shown). The purified lCD was G89, 987 nM; and G90, 219 nM (29). Peptides were prepared by bard’s algorithm were as follows: HA, 35 of these peptides for HLA-DR4 (as IC50) according to Roth-
tide (HA: 307-319) (Table 1). The predicted binding affinities matching the standard HLA-DR4IDR 1 helper epitope, HA pep-
the sequence was extended to include Pro N-terminal to either anchors for each HLA-DR Ag and the main P1 anchors for most class II alleles (Table 1). In peptides G88, G89, and G90, positions P3 and P4 are occupied by hydrophobic, aromatic, and aliphatic residues, in that order, to facilitate peptide binding in different frames.

The binding motifs of synthetic peptides may differ from those of natural ligands because the latter incorporate processing constraints in addition to binding requirements. Thus, peptides were synthesized by following, when possible, the common motifs for all MHC class II molecules defined by pool sequenc-
ing of naturally processed peptides (25). In F7, F13, and F14, the sequence was extended to include Pro N-terminal to either the Tyr (the P1 anchor for HLA-DR1, DR3, DR4, and DQ7) or the Trp (reportedly as the P1 anchor for HLA-DR4 and DR11). For comparative studies of the responses associated with HLA-
4, 13-mer analogues of Fl2, F7, and Fl3 (i.e., G88, G89, and G90, respectively) were selected, using the anchor alignment matching the standard HLA-DR4/DR1 helper epitope, HA peptide (HA: 307–319) (Table 1). The predicted binding affinities of these peptides for HLA-DR4 (as IC50) according to Roth-
bard’s algorithm were as follows: HA, 35 nM; G88, 180 nM; G89, 987 nM; and G90, 219 nM (29). Peptides were prepared by the Synthetic Antigen Laboratory of the M. D. Anderson Cancer Center, using a solid-phase method as described previously (17, 30). Their identity was determined by amino acid analysis. Their purity was 93–97% as determined by HPLC. Peptides were assigned by the Synthetic Antigen Laboratory.

Recombinant lCD. The lCD (Lys576–Val1255) was cloned by PCR from c-erbB-2 cDNA (provided by Dr. Jacalyn Pierce, National Cancer Institute). The lCD was expressed in Escherichia coli using a pET vector with an amino-terminal His tag. Recombinant lCD was purified from inclusion bodies by a combination of Ni2+ affinity chromatography, size exclusion, and ion exchange chromatography. The purified lCD was greater than 95% pure as judged by PAGE and Western analyses (data not shown).

close to the NH2 terminus, and other major but less essential anchors at P4, P5, P7, and P9, counting from the P1 anchor (26–28). Because many peptides are capable of binding to many different MHC class II molecules because their sequences contain overlapping binding motifs for MHC class II molecules (27, 28), each of the peptides synthesized contained at least two of three anchors for each HLA-DR Ag and the main P1 anchors for most class II alleles (Table 1). In peptides G88, G89, and G90, positions P3 and P4 are occupied by hydrophobic, aromatic, and aliphatic residues, in that order, to facilitate peptide binding in different frames.

Stimulation and Propagation of T Cells. Freshly harvested PBMCs from breast cancer patients and healthy volunteers were isolated by Ficoll/Hypaque centrifugation. CD4+ and CD8+ cells were isolated from the plastic nonadherent fraction with magnetic beads. Cells were cultured at 1 × 106 cells/ml in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) with 10% pooled human AB serum and antibiotics in 2 ml in each well of a 24-well plate (complete RPMI medium). HER-2 peptides were added at a final concentration of 25 
µg/ml. In other wells, PBMCs were stimulated with 25 µg/ml HA peptide, PHA at a final concentration of 1:100, or medium alone. After 6 days of stimulation with each peptide, cultures were expanded with IL-2 (Cetus) at 20 units/ml for the following week (17, 31). To induce Ag-specific T cells, the cells were then “rested” for 3–4 days by culture in the absence of IL-2. Then, the cells were stimulated at a 1:1 stimulator:responder ratio with irradiated (10,000 Rad) PBMCs and pulsed with individual peptides for at least 90 min at 37°C before addition to the cultures as described (17). For expansion, 4–5 days later, 20 units/ml IL-2 was added to the cultures for 7 additional days. Surface Ag expression was determined by fluorescence-acti-
vated cell sorting analysis using a FACSScan (Becton Dickinson, Sunnyvale, CA) with a log amplifier as described (17).

Proliferation Assays. For proliferation assays, a 100-µl aliquot was removed from each well of the 24-well plate of primary cultures after 4–6 days, as described (16). Quadruplicate samples were cultured in a 96-well plate with 1 µCi of [3H]Tdr in a final volume of 200 µl. The cells were harvested 16 h later, and the radioactivity was counted in a Beckman LS3501 liquid scintillation counter (16). A proliferative response was defined as positive when differences in cpm values between cultures that received peptides and cultures that did not receive peptides were significant by the unpaired Student’s t test (P < 0.05).

Cytokine Production. The ability to secrete IFN-γ, IL-4, and IL-10 was determined by culturing the PBMCs with the corresponding peptides. Supernatants were collected at different times and stored frozen at −20°C. The cytokine concentrations were measured by double sandwich ELISA using the corresponding kits provided by BioSource International (Cama-
rayo, CA). The cytokine assays were calibrated with human recombinant IFN-γ, IL-4, and IL-10 to detect each cytokine in the range of 15–1000 pg/ml. The following homeologous B cell lines were obtained from the American Society for Histocom-

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<th>Peptide code</th>
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<td>886–898</td>
<td>I K W M A L E S I L R R R</td>
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Table 1 HER-2 peptides used in this study
The Tyr and Trp italicized in position 3 or 4 may constitute P1 anchors. Similarly, the Val, Leu, and Met italicized in positions 4 and 5 may also constitute P1 anchors.
Fig. 1 Histograms of selected representative patterns of proliferation for eight breast cancer patients. A, patient 13, average NP value, 625 cpm; B, patient 7, average NP value, 862 cpm; C, patient 2, average NP value, 268 cpm; D, patient 9, average NP value, 272 cpm; E, patient 15, average NP value, 447 cpm; F, patient 12, average NP value, 313 cpm; G, patient 1, average NP value, 268 cpm; H, patient 5, average NP value, 568 cpm. Each determination was performed in quadruplicate; cpm for each of the replicates are represented by one triangle. Patients in A–F are HLA-DR4+ and those in G and H are HLA-DR4−. Responses to G89 were considered positive in A–D and F (HLA-DR4+ patients) and in G (HLA-DR4− patient) because in each of these donors tested, all cpm quadruplicate values in response to G89 were higher than each of the quadruplicate values of the control cultures unstimulated with peptide. Freshly isolated PBMCs from each donor were stimulated with peptides at a final concentration of 25 μg/ml. Responses were determined in 100-μl aliquots of cells removed from cultures on days 4–6 and tested for proliferation. Responses are shown for cultures stimulated for either 4 or 5 days. In most instances, significant proliferation was observed on two consecutive days (days 4 and 5 or days 5 and 6). An exception was made for patients 9 (D) and 15 (E), who showed responses only on days 5 and 6, respectively; patient 9 was considered a responder because the S.I. was >2.5, but patient 15 was considered a nonresponder because the S.I. to G89 was <2.0.

Results

Recognition of HER2 Peptides in Breast Cancer Patients. PBMCs of breast cancer patients were cultured with HER-2 peptides or medium alone for 4–6 days. To ensure that lack of responsiveness of PBMCs to any of HER-2 peptides did not reflect a generalized suppression of responses to Ag or mitogen, all subjects’ lymphocytes were tested for their ability to respond to PHA. Because responses to F7 (HER-2, 776–788) and F13 (HER-2, 884–899) were previously observed with higher frequency in healthy individuals (17), we wanted to address the question of the ability of the T cells from breast cancer patients to respond to HER-2 peptides, in association with certain MHC class II types, expression of HER-2 in their primary tumor, and the lymph node status. Because the patients were tested in the order they presented and not based on their HER-2 expression, when the S.I. was <2.0, to increase the sensitivity of detection, responses were considered positive when the cpm in each of the quadruplicate cultures stimulated with peptides was higher than each of the quadruplicate control cultures on two consecutive days (days 4 and 5 or days 5 and 6). This approach allowed us to identify responders without arbitrary cutoffs, using S.I.

Emphasis was given to HLA-DR4+ patients because of the recently reported association of HLA-DR and HLA-DR4 with...
The results (Fig. 14) show that responses to control peptide 088 counterparts F7 and Fl3 were tested in the same experiment. Another patient (No. 13). Both peptides G89 and 090 and their control peptide G88 and NP, the experiments were repeated with stimulated with 088. With 089 were not higher than the cpm values in cultures replicate cultures stimulated with the control 088 peptide of added peptide (NP). In these six patients, the cpm values in each replicate in PBMC cultures from the was confirmed by the fact than in the two nonresponders (Fig. 1, Fig. 1. In 450-462) based on the sequence of Fl2 (HER-2, 449-464) was and G), the cpm values in all replicate cultures were higher than significant followed by a hydrophobic aromatic residue at P3 and P4, (Table 1). A control peptide of the same length: 088 (HER-2, 450-462) based on the sequence of Fl2 (HER-2, 449-464) was prepared (Table 1) because it had the same pattern of residues in P3 and P4 as G89 and G90. G88 was chosen as a control because responses to F12 were previously observed only infrequently (17). The predicted HLA-DR4 binding affinity of G88 was similar to that of G90 but significantly lower than that of G89.

The responses to HER-2 peptides G89 and G90, together with the responses to control peptide G88 for 8 of the 18 patients tested (including responders and nonresponders), are shown in Fig. 1. In all six G89 responding patients shown (Fig. 1, A–D, F, and G), the cpm values in all replicate cultures were higher than the cpm values in each replicate in PBMC cultures from the same patient that had not been stimulated with exogenously added peptide (NP). In these six patients, the cpm values in the replicate cultures stimulated with the control G88 peptide of higher binding affinity to HLA-DR4 than G89 were not significantly different from the cpm values in the NP cultures. This was confirmed by the fact that in the two nonresponders (Fig. 1, E and H), the cpm values in the replicate cultures stimulated with G89 were not higher than the cpm values in cultures stimulated with G88.

To verify the stimulatory ability of G89 in comparison with control peptide G88 and NP, the experiments were repeated with another patient (No. 13). Both peptides G89 and G90 and their counterparts F7 and F13 were tested in the same experiment. The results (Fig. 2A) show that responses to control peptide G88 were not significantly different from the NP cultures. Responses to G89 and G90 were significantly different from responses to NP or G89, but they were not significantly higher than responses to F7 and F13. The ability of PBMCs of HLA-DR4+ healthy volunteers to recognize HER-2 peptides was also tested. Significant proliferative responses by the same criteria were detected in HLA-DR4+ individuals after primary stimulation of PBMCs with various HER-2 peptides, of which responses to two donors (No. 2 and 3) are shown (Fig. 2, B and C). CD4+ cells responded to G89 (Fig. 2C). Thus, the ability to recognize sequences of the HER-2 protein is within the realm of the T-cell receptor of healthy volunteers, as we reported (17).

Proliferative responses to HER-2 peptides from all 18 patients tested are summarized in Table 2. G89 and F7 were recognized by PBMCs from 10 and 8 patients, respectively. PBMCs from six patients recognized both G89 and F7. Responses to G90 and F13 were observed in six and three patients, respectively. The results show a higher frequency of responses for G89 and G90 containing MHC class II anchors in P3 and P4 than for their analogues (F7 and F13) with the anchors shifted. The frequency of responses to G89 was significantly higher than to other peptides (P = 0.02). The results also show preferential association (7 of 9) of the responses to G89 with the presence of HLA-DR4 (P = 0.01). Of the other alleles that were represented, four of five HLA-DR3 patients responded to G89, but three of four responders were also HLA-DR4+. Four of six HLA-DR2+ patients responded to G89, but three of four responders were also HLA-DR4+. The other three HLA-DR4+ responders responded preferentially to F13, with S.I. values of 1.9, 1.7, and 1.5, respectively, but no significant proliferation to G89 was observed (data not shown).

HER-2 staining for the autologous breast tumors was performed by immunocytochemistry. HER-2 was overexpressed favorable prognosis in breast cancer (33, 34). Because both F7 and F13 contain HLA-DR4 anchors but differ in the length and position of the anchor motifs, we synthesized two 13-mers, designated G89 and G90. Each contained a hydrophobic aromatic followed by a hydrophobic aliphatic residue at P3 and P4 (Table 1). A control peptide of the same length: 088 (HER-2, 450-462) based on the sequence of Fl2 (HER-2, 449-464) was prepared (Table 1) because it had the same pattern of residues in P3 and P4 as G89 and G90. G88 was chosen as a control because responses to F12 were previously observed only infrequently (17). The predicted HLA-DR4 binding affinity of G88 was similar to that of G90 but significantly lower than that of G89.

The other three HLA-DR4- donors responded preferentially to G89 and 090 were significantly different from responses to F7 and F13. The ability of PBMCs of HLA-DR4+ healthy volunteers to recognize HER-2 peptides was also tested. Significant proliferative responses by the same criteria were detected in HLA-DR4+ individuals after primary stimulation of PBMCs with various HER-2 peptides, of which responses to two donors (No. 2 and 3) are shown (Fig. 2, B and C). CD4+ cells responded to G89 (Fig. 2C). Thus, the ability to recognize sequences of the HER-2 protein is within the realm of the T-cell receptor of healthy volunteers, as we reported (17).
Fig. 3 A, specificity of proliferative responses of G89R T cells (derived after expansion in IL-2 of primary stimulated PBMC from patient 13). PHA blasts from patient 13 were used as autologous APC. B, specificity of proliferative responses of G89L and G90L (derived from donor 3). The G90L line was developed by priming with 090. Autologous plastic-adherent PBMCs were used as APC.

Table 2  Summary of proliferative responses of breast cancer patients to HER-2 peptides

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% positive  22.2  33.3  11.1  44.4  16.7  55.6  22.2

only in tumors from patients 6 (DR4-) and 13 and 15 (DR4+). The disease status, tumor size, lymph node status, tumor grade, and HER-2 expression were also compared with the response to these peptides. There was no correlation between proliferative responses and these clinical parameters.

No apparent correlation was found between HER-2 overexpression and proliferative responses to HER-2 peptides. Of the three HER-2 patients (two DR4+ and one DR4-), responses to G89 were seen only in patient 13 (DR4+). These responses were stable on both day 4 and day 5 of testing. In contrast, patient 15 responded only to F13 (on day 6) (Fig. 1E), whereas patient 6 (DR4-) responded only to G88 and F7. Of the seven HER-2 patients, five showed responses to G89 on at least two consecutive days of assay, one showed a response on day 5 (S.I. >2.5) (Fig. 1D), and one failed to respond. This group was too small for statistical analysis to address possible differences in response due to HER-2 overexpression and disease progression.

Analysis of the significance and specificity of responses focused on characterizing the proliferative and cytokine response to G89 by T cells from patient 13 (HER-2+). To address the specificity of proliferative responses, G89-primed PBMCs from patient 13 (Fig. 2A) were expanded in culture with IL-2 and restested for their proliferative responses to G88, G89, and G90. Freshly isolated monocytes/macrophages were not available from this patient for restimulation and follow-up studies. We used PHA blasts from the same patient as APC. Results in Fig. 3A show that G89-stimulated cultures (G89R) recognized G89 significantly better than G88 and G90, but the overall level of response was low. In fact, no clear improvement in proliferative activity was seen after expansion in IL-2 without restimulation with peptide. Because this may be due to the poor APC ability of PHA blasts, the specificity of G89 stimulation was characterized in parallel with the response from donor 3 (also HLA-DR4+) because autologous APC, plastic-adherent cells were available. Cultured G89-primed PBMCs of this donor (designated G89L) showed significantly higher proliferative responses to G89 than to control G88 peptide at restimulation...
when presented on autologous plastic-adherent fraction of PBMCs (Fig. 3B). In contrast, the corresponding G90-primed (G90L cells) showed significantly lower specific proliferation to recall with G90 than G89L cells to recall with G89, suggesting that G90 may prime the T cells for a cross-reactive epitope.

**Secretion of IFN-γ by G89-stimulated T-Cell Lines.**
Because the levels of IFN-γ and IL-4 in primary cultures were either very low or undetectable (data not shown), studies were conducted on secondary cultures. Recent studies have shown that IL-2 is required for Th2 differentiation and IL-4 production (35). To determine the type of cytokine responses to G89, cultures were established in low-dose IL-2 after initial stimulation of PBMCs with G89 from patient 13 (G89R) and donor 3 (G89L) and with G90 from donor 3 (G90L). The ability of these cells to secrete IFN-γ, IL-4, and IL-10 in response to the priming peptide was tested in parallel with the control peptide G88.

In preliminary experiments, we observed that the levels of IL-4 and IL-10 in response to G89 were low or undetectable. Because secretion of IL-4 and IL-10 may be delayed or HER-2 peptide G89 may be less efficient in inducing Th2 cytokines, we first determined the cytokine profile in response to G89 and G90 at both 40 and 140 h. The peptide G88 was used as control. The G89R T cells secreted high levels of IFN-γ in response to G89 but not in response to control G88 peptide (Fig. 4). These cells also secreted significantly less IL-4 and IL-10 than IFN-γ after either 40 or 140 h in culture, suggesting a preferential Th1 or Th0-Th1 response to G89. However, because the responses determined were obtained with short-term bulk cultures and because background levels of IL-4 and IL-10 were present, we defined this reactivity as Th0-Th1. The G90-primed G90L T cells showed significant cross-reactivity with G88 with regard to IFN-γ and IL-10 secretion and secreted significantly more IL-10 than did G89R G89-induced T cells. Although the levels of IFN-γ in response to either G90 or G88 were higher than the levels of IL-4, the levels of IL-10 were higher than the levels of IFN-γ. Although it is possible that earlier levels of IFN-γ secreted in response to G90 may have been higher, the very high levels of IL-10 may suggest the presence of nonspecific Th2 cells activated following the initial G90 stimulation.

To address whether IFN-γ was secreted in response to G89 and the ICD (which contains this peptide but not the control G88 peptide), in association with HLA-DR4, G89R and G89L T cells were tested for cytokine secretion in response to G89 presented by PBMCs of different phenotypes (Table 3). Comparison of MHC I phenotype between APC and responders suggested that the IFN-γ was not secreted in response to MHC I. G89L shared HLA-A2 and HLA-B44 with APC from donor B. Although this may raise the possibility that G89, G88, or shorter fragments can be presented by HLA-A2, APC from donor C also shared HLA-A2 with G89L and expressed HLA-23, B41, and B46. However, the levels of IFN-γ secreted were low compared with the levels detected when G89 was presented by APC from donors A and B. Thus, although the possibility that MHC I molecules may present G89 cannot be excluded, comparison of the MHC I phenotypes suggest that G89-stimulated T cells secrete cytokines in response to MHC II molecules.

Significantly higher levels of IFN-γ than IL-4 were se-
creted by G89L cells in response to G89 and ICD when G89 was presented by autologous APC to G89L. A similar pattern of response was observed for the G89R cells obtained from the breast cancer patient. The IFN-γ response by both G89L and G89R to the control G88 peptide, although significant, was at least one order of magnitude lower than to G89, or to the ICD. In this experiment, APC (from donor 3) and responders shared only HLA-DR4 and HLA-DQ6. Thus, in the presence of APC that shared either (a) DR15 and DQ6 with G89L, (b) DQ6 with G89L, or (c) DQ6 with G89R, significant levels of IFN-γ were observed in response to G89 but not to ICD, by both responders, the healthy donor and the cancer patient. The results also show that the restriction element used by G89L and G89R for recognition of exogenously added peptides is not exclusively HLA-DR4. These results suggest that it is likely that a naturally processed peptide from HER-2 is recognized by HER-2 peptide G89-primed T cells in the context of HLA-DR4.

IFN-γ and IL-4 release was observed in response to G89 (but not to ICD) presented by APC sharing either HLA-DR15 or HLA-DQ6 or both with the responders. These levels of cytokines were not induced in response to G88. Although the levels of cytokines secreted when G89 was presented by other HLA molecules were lower than levels in response to HLA-DR4, a certain pattern of “promiscuous” recognition was present, consisting always of higher levels of IFN-γ than IL-4. This suggests that although MHC class molecules of DR4-APC could present exogenously loaded G89 in a form recognizable to G89L and G89R T-cell receptor, the naturally processed and presented fragment of the ICD may have been derived from the one presented by DR4. A similar pattern of responses by G89L and G89R, although with reduced IFN-γ levels, was seen using lymphoblastoid cell lines WT51 (homozgous for DR4) and E4428342 (homozgous for DR15) as APC (data not shown).

**DISCUSSION**

In this report, we present evidence that PBMCs from primary breast cancer patients respond by proliferation in vitro to a number of HER-2 peptides. The responding population consists of CD4+ cells, as demonstrated in a previous study (16) and as suggested by the ability of the responding cells to secrete IFN-γ in response to these peptides when presented by MHC class II. In previous experiments, we noted that anti-MHC II antibodies and, to a lesser extent, anti-MHC I antibodies inhibited proliferation of PBMCs to helper peptides (17). Low levels of proliferation compared with CD4+ cells were observed with isolated CD8+ cells in a healthy donor, but the differences in cpm between G89-primed (G89L) and G90-primed CD8+ cells from donor 3 were significant. However, given the length of these peptides, the stimulatory potential for CD8+ cells after the binding of G89 to certain HLA class I allele products deserves further investigation. The frequency of the responses was higher for G89 (56%) than for the other peptides tested, suggesting that G89 may represent an immunodominant epitope in the group analyzed. Of interest, the responses to G89 appeared to associate more frequently with the presence of HLA-DR4 (in seven of nine cases), suggesting that HLA-DR4 may be the presenting element.

The fact that F7 and G89 are equal in length and differ by one residue at their NH2- and COOH-terminal ends suggests that the epitope formed by G89 in vitro, when used at a concentration of ~10 μM, is specifically recognized. The frequency of responses appeared not to be related to the binding affinity of these peptides to DR4. The predicted binding affinity of G89 to HLA-DR4 was significantly lower than that of peptides G88 and G89 of the same length.

It is unknown at this time whether for G89, binding to HLA-DR is sequence specific, is restricted to certain DR4 subtypes, or is promiscuous. Depending on which P1 frame is used, Tyr or Trp can serve as an anchor for DRB1*0401 but not for *0404 and *0402. Similarly, at P4, negatively charged residues Asp and Glu are accepted by DRB1*0401 and *0404 but not by *0402, which accepts positively charged residues, such as Lys/Arg (36). This suggests that G89 (as well as G90) may preferentially bind to different DR4 subtypes and use alternative binding frames (i.e., with Val, Leu, and Met for the P1 frame). Additional studies are required to address the ques-
tion of whether the antigenicity of G89 is associated with the predicted poor binding in a fashion similar to that reported for most tumor peptides from self Ag recognized by human CTLs.

T-cell cultures primed with G89 responded at restimulation by secreting more IFN-γ than IL-4 and IL-10, suggesting the preferential activation of a Th1 response. Because the experiments were performed with bulk cultures and not with clones, and because IL-4 and IL-10 were detectable, we would rather define this reactivity as Th0-Th1. This response was apparently not directed to a cryptic HER-2 epitope because peptide-primered cells recognized the ICD. The IFN-γ response to ICD of G89-primed T cells suggests that HLA-DR4 may be the presenting element for a naturally processed epitope similar in structure to G89.

Recent studies to examine proliferative responses in breast and ovarian cancer using HER-2 peptides of various lengths and randomly selected patients who had not been HLA-typed showed T-cell responses to several HER-2 peptides (16, 17). One of those, defined as p783 (HER-2, 776–797), reportedly activated responses of T cells to the HER-2 protein in a breast cancer patient (16). Although the magnitude of G89-induced responses was significantly lower than that reported for p783, our results indicate a trend of increased proliferation to G89. F7 (HER-2, 776–788) was also found to induce T-cell proliferation in both healthy donors and ovarian cancer patients (17). The data in this study suggest that within the area HER-2, 776–797, nests a dominant HER-2 epitope for CD4+ cells. Because HLA-DR4 is expressed in approximately 25% of humans, this epitope may be an important peptide for activation and regulation of T-cell differentiation toward a Th1 response. It may also be beneficial for CTL activation and expansion.

The observation that T cells from both healthy donors and patients whose tumors overexpress HER-2 can respond to G89 argues against the induction of tolerance to this epitope and/or against autoimmune activation of G89-specific T cells by HER-2 only after protein overexpression. In both this and the previous study with p783 (16), the proliferative responses were observed early, 4–6 days after stimulation. This may argue against a primary response to G89. Primary in vitro responses of T cells to some foreign Ag have been shown to require a significantly longer time (7–9 days) to be detected as significant proliferation (37, 38), although it is unknown whether these findings can be extended to self Ag. The low levels of cytokines at primary stimulation may even argue against a recall response, unless the frequency of G89-specific cells is very low. Additional studies are needed to clarify this point. A possibility that needs to be considered is that epitopes such as G89 may induce in vivo a limited number of Th1 cells, which may exert a regulatory function. Preliminary studies in our laboratory show that primary stimulation of T cells from healthy donors with either F7 or F12 or F13 followed by culture in IL-2 leads to preferential expansion of F7-responsive cells. These cells secreted high levels of IFN-γ at secondary and tertiary stimulation with F7. This pattern of responses suggests a determinant spreading effect as described for some cryptic epitopes (39).4

Previous studies of HER-2 focused primarily on characterizing CTL epitopes (40, 41). The observation, in different systems, that human tumors are antigenic although poorly immunogenic emphasizes the need for development of approaches to induce and augment an immune response to tumor. Although in vitro and in vivo models show that induction of tumor-specific CTLs can be achieved by costimulation (42), the observed activation of Th1 response by the same tumor Ag recognized by CTLs suggests an involvement of CD4+ cells in the reaction to tumor. It also raises the question of whether the G89-induced Th0-Th1 response plays a protective role during tumor spread or whether it is down-regulated by Th2 cytokines subsequent to recognition of other peptides after HER-2 overexpression. In this context, the characterization of epitopes that regulate Th1 responses, which can in turn control the spread of Th1/Th2 responses by other self peptides, may have important implications not only for CTL induction but also for understanding the regulation of human tumor immunity.

ACKNOWLEDGMENTS

We thank Susan Mondragon for expert editorial assistance.

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Proliferative Responses to HER-2 Peptides


Proliferative and cytokine responses to class II HER-2/neu-associated peptides in breast cancer patients.

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