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A Chimeric Multi-Human Epidermal Growth Factor Receptor-2 B Cell Epitope Peptide Vaccine Mediates Superior Antitumor Responses

Naveen K. Dakappagari,* John Pyles,† Robin Parihar,‡ William E. Carson,§ Donn C. Young,¶ and Pravin T. P. Kaumaya*†‡¶

Immunotherapeutic approaches to cancer should focus on novel undertakings that modulate immune responses by synergistic enhancement of antitumor immunological parameters. Cancer vaccines should preferably be composed of multiple defined tumor Ag-specific B and T cell epitopes. To develop a multiepitope vaccine, 12 high ranking B cell epitopes were identified from the extracellular domain of the human epidermal growth factor receptor-2 (HER-2) oncoprotein by computer-aided analysis. Four novel HER-2 B cell epitopes were synthesized as chimeras with a promiscuous T cell epitope (aa 288–302) from the measles virus fusion protein (MVF). Two chimeric peptide vaccines, MVF HER-216–339 and MVF HER-2485–503 induced high levels of Abs in outbred rabbits, which inhibited tumor cell growth. In addition, Abs induced by a combination of two vaccines, MVF HER-216–339 and MVF HER-2628–647 down-modulated receptor expression and activated IFN-γ release better than the individual vaccines. Furthermore, this multiepitope vaccine in combination with IL-12 caused a significant reduction (p = 0.004) in the number of pulmonary metastases induced by challenge with syngeneic tumor cells overexpressing HER-2. Peptide Abs targeting specific sites in the extracellular domain may be used for exploring the oncoprotein’s functions. The multiepitope vaccine may have potential application in the treatment of HER-2-associated cancers. The Journal of Immunology, 2003, 170: 4242–4253.

Human epidermal growth factor receptor-2 (HER-2), a member of the epidermal growth factor receptor family, is overexpressed at high levels in a wide variety of human cancers, including breast, ovarian, colon, gastric, prostate, and lung malignancies (1). HER-2 overexpression correlates directly with tumor aggressiveness and decreased patient survival (2, 3) and confers resistance to certain drugs, hormones, and radiation therapies (4–6); therefore, it is considered an attractive target for active specific immunotherapy (7). mAbs capable of down-modulating the receptor expression are known to reverse the transformed phenotype and inhibit the growth of experimental tumors (8, 9). One such mAb, 4D5 or its humanized counterpart, hereceptin, directed against the extracellular domain of HER-2 has been used to treat HER-2-associated cancers effectively in both animal models and cancer patients (10, 11). While passive infusion with such tumor inhibitory mAbs has shown good clinical efficacy, several important considerations, e.g., generation of anti-idiotypic Abs, inadequate tissue distribution, and levels necessitating multiple infusions and hence the associated cost, limit the utility of passive immunotherapy protocols.

In contrast, vaccination strategies designed to elicit endogenous tumor inhibitory Abs and stimulate immunologic memory may be more advantageous for providing long-term benefits to the patient at a much-reduced cost. These vaccines may be applied either for treatment of metastatic disease or to contain minimal residual disease following resection of solid tumors. Overexpression of HER-2 on tumor cells results in weak, but detectable, levels of HER-2-specific Abs and T cells in early stage breast cancer patients without any signs of autoimmune disease (7, 12, 13). The lack of an autoimmune response toward normal cells expressing basal levels of HER-2 may imply that a threshold (higher) level of HER-2 expression, such as that present on a tumor cell, is required for initiating an immune attack. These observations raise the possibility of augmenting the weak pre-existing immunity to therapeutic levels through appropriate vaccination strategies designed to boost cross-reactive immunity to the tumor Ag.

Immunotherapeutic approaches for the treatment of HER-2-associated cancers using whole protein vaccines administered in the form of vaccinia viruses, tumor cells, DNA, or recombinant proteins have been successful in generating a protective immune response against tumor Ags in murine models (14–17). These studies suggest that stimulation of the immune response by a complex vaccine harboring multiple epitopes results in an effective antitumor response involving several mechanisms. Thus, active specific immunotherapeutic strategies targeting a single epitope may not be as effective as a complex vaccine. A combination vaccine approach that focuses on both the best cellular and Ab epitopes would probably be the most effective way to elicit tumor protective immunity. In addition, the use of multiple chimeric peptides incorporating promiscuous T cell epitopes may strengthen the level of the immune response and polarize Th cell activity.

A need for epitope-based vaccines stems from the fact that tolerance to self-Ags, e.g., HER-2, may limit a functional immune
response to whole protein-based vaccines due to activation of suppressor T cells that maintain tolerance to host Ags or alternate regulatory mechanisms (18). Indeed, immunization of mice with vaccinia viruses encoding either the entire rat neu (homologue of HER-2) or neu extracellular domain induced both T cell and Ab responses that protected mice from subsequent challenge with neutransformed NIH-3T3 cells. However, this strategy was ineffective in eliciting an immune response in rats (19). In contrast, effective humoral and Th responses could be generated against new oncoprotein in rats immunized with Th epitope peptides derived from the rat neu protein, but not by vaccination with the cognate protein itself (20). The assumption in these studies was that the Th epitope peptides correspond to subdominant epitopes that activate unedited self-reactive T cells. It is also likely that the Th peptides elicited cross-reactive immunity to the autologous protein. These studies suggest that tolerance to self-protein can be overcome by certain parts of the protein that can selectively activate the immune system.

The identification of epitopes on HER-2 that are either stimulatory or inhibitory is critical to the development of strategies to better manipulate the Ab response for therapeutic benefit. By a proper selection of epitopes from specific regions of HER-2 capable of inducing tumor inhibitory Abs and by careful elimination of epitopes that stimulate cancer cell growth, candidate vaccines can be identified that could provide beneficial effects. This is especially important because immune responses elicited by whole protein vaccines can stimulate the growth of tumor cells when a subclass of protein Abs mimics the activity of growth factor ligands. Several Abs capable of stimulating the phosphorylation of HER-2 and subsequent growth of HER-2-bearing tumor cells have been described (21, 22).

Studies performed in both animal models (20, 23–25) and human cancer patients (26–28) clearly demonstrate that epitope-based peptide vaccines are effective in eliciting immunity in the context of self and have the potential to selectively kill HER-2-overexpressing tumor cells. We have previously reported that a chimeric peptide vaccine incorporating a promiscuous Th cell epitope and a HER-2B cell epitope, measles virus fusion protein (MVF) HER-2-628–647 can prevent spontaneous mammary tumor development in HER-2/neu transgenic mice (24). We reasoned that a combination of B cell epitopes selected based on their ability to inhibit tumor growth would exert synergistic anti-tumor effects and confer superior protection compared with a single B cell epitope vaccine. To address this hypothesis, we evaluated the tumor inhibitory potential of four novel HER-2 B cell epitopes identified by computer-aided analysis and synthesized as chimeric constructs with the promiscuous T cell epitope (aa 288–302) from the MVF protein.

We report here the identification of two new HER-2 B cell epitopes (316–339 and 485–503) that induce high titrated Abs capable of inhibiting the growth of human breast cancer cells. We also demonstrate that Abs induced by a combination of two distinct B cell epitope constructs, MVF HER-2-316–339 and MVF HER-2-628–647 down-modulated receptor expression and activated IFN-γ release better than either of the individual B cell epitope-specific Abs. Furthermore, immunization of mice with a combination of this multiepitope vaccine and IL-12 caused a significant reduction in the number of lung metastases.

Materials and Methods

Prediction of B cell epitopes

The selection of candidate B cell epitopes expressed within the human HER-2 extracellular domain (ECD) was accomplished by computer-aided analysis based on various correlates of protein antigenicity as reviewed by Kaumaya et al. (29). Briefly, 1) hydrophilicity profiles were generated by the program of Hopp and Woods and Parker et al. (30, 31); 2) protrusion index values were calculated by the method of Janin (32), which predicts the surface-exposed parts of the protein based on the accessibility of the amino acids to the solvent, and also by the method of Novotny et al. (33), which predicts protein surfaces accessible to Ab binding using large (1-nm) sphere probes; 3) hydrophobicity (affinity for water) profiles were calculated using the scale of Kyte and Doolittle (34); 4) the probability that a seven-residue sequence is antigenic was determined by the method of Welling et al. (35); and 5) bulk hydrophobic profiles were generated based on the rules proposed by Manavalan and Ponnuswamy (36). All protein profiles were generated using a seven-residue window moving along the protein sequence. The profiles were normalized for the purpose of comparing different methods. The mean hydrophilicity (or accessibility, etc.) over the entire HER-2 ECD protein sequence was calculated, and a zero value was set at the average. Maximum and minimum hydrophilicity (or accessibility, etc.) values were set on a scale of +4 to −4. One hundred and forty-four sequences (10–30 residues long) were then ranked by comparing the joint predictions of 10 algorithms (hydrophilicity, accessibility, etc.). The highest ranking sequences had the highest individual score for the analyses examined (10/10), and successive candidates had the next highest score (9/10), etc. The best-scoring epitopes were further ranked by correlation with their secondary structural attributes, e.g., an amphiphilic α-helical sequence is preferred over a random coil fragment. Computer programs by Chou and Fasman (37) were used to predict the secondary structure. Consideration was given to the individual amino acid sequence (e.g., hydrophobic/hydrophilic balance, aromaticity). Finally, electrostatic ion pairs and helix dipole interaction in helical segments were also considered.

Synthesis and characterization of chimeric peptides

Four HER-2 B cell epitopes, 27–45, 316–339, 485–503, and 605–622, were synthesized co-linearly with Th epitope, aa 288–302 from MVF and a four-residue amino acid linker (GPGL), as previously described (38) on a Milligen/Biosearch 9600 peptide synthesizer (Bedford, MA). The crude peptides were purified by reverse phase HPLC, and the identity of the peptides was determined by electrospray ionization spectrometry (C Campus Chemical Instrumentation Center, Ohio State University, Columbus, OH). Mass spectrometry indicated that the methionines in the sequences 27–45 and 625–622 were oxidized during or after cleavage of the peptides from the resin (MVFHER-2 27–45 (M = 4453, Found 4469, 4484); MVF HER-2 (M = 4463, Found 4478). Mass spectrometry profiles of MVF HER-2-316–339 and MVF HER-2-625–622 indicated calculated M values of 4953 and 4598, respectively. Methionine sulfone(s) are known to perturb the secondary structure and biological function of proteins (39). Sequence 27–45 has two methionines buried in α helices, and sequence 605–622 harbors one methionine in the β sheet. Therefore, these two immunogens were dissolved in 3–5% acetic acid at 5 mg/ml and were reduced using 2.8 M N-methylmercaptoacetamide at 37°C for 40 h as described by Houghten et al. (40). Mass spectrometry of HPLC-purified peptides indicated that these peptides were completely reduced. The peptides were >95% pure before immunization.

Energy minimization

Energy minimization calculations on the cysteine cross-linked B cell epitopes were performed using Hyperchem molecular modeling software (Release 5.0; Hypercube, Waterloo, Ontario, Canada). All calculations were performed in AMBER force field with values set at default. A total of 500 cycles were performed using the Polak-Ribere algorithm.

Circular dichroism (CD) measurements

CD spectra were obtained on an AVIV model 62A DS CD instrument (Lakewood, NJ). All spectral measurements were performed at 25°C under continuous nitrogen purge of the sample chamber, using a 0.1-cm path length quartz cuvette. Peptides were dissolved in water or 50% trifluoroethanol (TFE) at a concentration of 100 μM. The mean residue ellipticity ([θ]MME) values were calculated according to the equation: [θ]MME = (θ × 100 × M)(n × c × l), where θ is the recorded ellipticity (deg), M is the molecular mass of the peptide, n is the number of residues in the peptide, c is the peptide concentration (milligrams per milliliter), and l is the path length of the cuvette. The helicity of peptides was determined according to Chen et al. (41) with reference to mean residue ellipticity of polylysine for 100% α helix (θααα = −35,700 (42).
Peptide immunization and cytokines treatments

All animals were purchased from Harlan (Indianapolis, IN). Pairs of New Zealand White outbred rabbits were immunized in the thigh muscle with a total of 1 mg of chimeric peptides dissolved in PBS with 100 μg of a muramyl dipeptide adjuvant (N-acetyl-glucosamine-3-yl-acetylt-1-alanyl-o-isoglutamin) and emulsified (50/50) in squalene/aracel A vehicle (4/1). A total of four booster injections were given with 500 μg of emulsified peptides every 4 wk thereafter. Sera were collected, and complement was inactivated by heating to 56°C for 30 min. High titer sera were purified on a protein A/G agarose column (Pierce, Rockford, IL), and eluted Abs were concentrated and exchanged in PBS using 100-kDa cutoff centrifuge filter units (Millipore, Bedford, MA). The concentration of Abs was determined with the Coomassie Plus protein assay reagent kit (Pierce).

on a protein A/G agarose column (Pierce, Rockford, IL), and eluted Abs were concentrated and exchanged in PBS using 100-kDa cutoff centrifuge filter units (Millipore, Bedford, MA). The concentration of Abs was determined with the Coomassie Plus protein assay reagent kit (Pierce). Groups of five IRC strain outbred mice, each 6–8 wk old, were immunized s.c. in the flank with 100 μg of peptides and 100 μg of nor N-acetyl-glucosamine-3-yl-acetylt-1-alanyl-o-isoglutamin, prepared as described for rabbit immunization. Mice receiving a combination of peptides were immunized separately with 100 μg of each peptide in separate flanks. The same dose of booster injections was administered twice after 3 and 6 wk. Sera were collected by retro-orbital bleeding 2 or 3 wk after each immunization for determination of Ab titers. For tumor challenge studies, groups of six BALB/c mice (6–8 wk old) were administered IL-12 (1 μg/mouse i.p.; gift from Genetics Institute, Cambridge, MA), IFN-α/β (1000 IU/mouse), or the cytokine diluent (PBS plus 1% BALB/c mouse serum) on the day before, day of, and the day after each peptide immunization in a final volume of 0.2 ml. All peptide immunizations were performed according to the schedule described above for IRC mice.

ELISA

This assay was performed as previously described (24). Ab titers were defined as the reciprocal of the highest serum dilution with an absorbance of 0.2 or greater after subtracting the background. All data represent the average of duplicate samples.

Mouse isotyping

All BALB/c mouse sera from tumor challenge studies were typed using a Mouse Typer Subisotyping Kit (Bio-Rad, Hercules, CA). The assay was performed according to the manufacturer’s instructions, except that a 1/1000 dilution of goat anti-rabbit IgG HRP conjugate was used.

Cell lines and Abs

All cell culture media, FCS, and supplements were purchased from Life Technologies (Grand Island, NY). The human breast tumor cell lines, SK-BR-3 (HER-2 overexpresed), BT-474 (HER-2 overexpressed and 2×105 molecules/cell) and BT-474 (HER-2 overexpressed, 10,000–50,000 molecules/cell), and MDA-468 (HER-2 overexpressed) were purchased from American Type Culture Collection (Manassas, VA) and maintained according to the supplier’s guidelines. HER-2 mAbs, Ab-2 (clone 9G6), Ab-15 (clone 3B5), and Ab-16 (clone L26) were purchased from Neomarkers (Fremont, CA). Humanized mouse mAb, herceptin, was provided by Genentech (San Francisco, CA).

Flow cytometry

This assay was performed as previously described (24).

Immunoprecipitation and Western blotting

This assay was performed as previously described (24).

Cell growth inhibition assay

MCF-7 and BT 474 cells were plated at 10,000 and 20,000 cells/well, respectively, in triplicate as determined by the dose-response curves and allowed to adhere for 24 h. The culture medium was replaced with 1.5% FCS-containing culture medium. After another 24 h, peptide Abs and normal rabbit Ig (negative control) were added at 100 μg/ml in triplicate. The Ab concentrations (100 μg/ml) selected for this assay were found to optimally inhibit tumor growth based on preliminary dose-response studies. For Ab-blocking experiments, the Abs were preincubated with corresponding immunogens at an Ab to Ag molar ratio of 1:20 for 1 h at 37°C before their addition to the cells. The plates were incubated for 72 h at 37°C, after which the number of viable cells was determined indirectly by measuring the color change obtained by the bioreduction of MTT at 570 nm as detailed by Hansen et al. (43). Results are expressed as the percent inhibition (normal rabbit Ig – peptide Ab/normal rabbit Ig × 100).

Receptor down-modulation

This procedure was adapted from the report by Klapper et al. (9). Briefly, tumor cells were incubated with antipeptide sera for 2 h, and the level of HER-2 molecules that remained at the cell surface was determined by binding of an 125I-labeled Ab, herceptin. Herceptin was radio labeled with Na125I (Amersham Pharmacia Biotech, Piscataway, NJ) by the chloramine T method. The percent receptor down-modulation was calculated as (preserum – antipeptide serum/preserum) × 100.

IFN-γ release

Ninety-six-well plates were coated with 50,000 BT474 or MDA-468 human breast cancer cells. After 24 h the culture medium was removed, and peptide vaccine-elicited mouse sera or preimmune serum were added at a 1/10 dilution; herceptin and human IgG were added as controls at 10 μg/ml for 2 h to coat the tumor cells. Wells were washed of any uncoated Ab, and 2×105 purified human PBMCs (American Red Cross, Columbus, OH) were added to each well and cultured in presence of 10 ng/ml recombinant human IL-12 (Genetics Institute). Control wells contained tumor cells and PBMCs supplemented with IL-12 alone. After 3 days, cell-free supernatants were harvested, and IFN-γ levels were determined by a sandwich ELISA.

Tumor cell challenge and evaluation of lung metastases

Mouse renal carcinoma cells (Renca-luc/ZerhB-2) transfected with lucZ and human HER-2 were a gift from Dr. W. Wels (Chemotherapeutisches Forschungsinstitut, Frankfurt am Main, Germany). These tumor cells syn- geneic to BALB/c mice were maintained in culture as described previously (44). Two weeks after the final immunization, each mouse was injected with 105 tumor cells through the lateral tail vein in 0.25 ml of PBS. Lungs from all mice were harvested 28 days after tumor cell challenge and fixed overnight at 4°C in PBS containing 2% formaldehyde and 0.2% glutaraldehyde. The fixative solution was removed, and the lungs were washed in PBS and stained overnight at 37°C in the dark with 5-bromo-4-chloro-4-indolyl-β-D-galactopyranoside (X-gal) solution containing 4 mM K4Fe(CN)6, 4 mM K2Fe(CN)6, 3H2O, 2 mM MgCl2, and 1 mg/ml X-gal in dimethylformamide. All reagents were purchased from Sigma-Aldrich (St. Louis, MO). Dark blue pulmonary metastases were enumerated in a blinded fashion by R.P. and N.K.D using an illuminated magni- fier. A maximum of 200 lung metastases could be enumerated, beyond which the lungs appeared fully saturated and were given a value of 250 for the purpose of statistical analysis.

Statistical analysis

Pulmonary metastases enumerated in various groups of animals were analyzed by two-way ANOVA with Dunnett’s test (45), in which the control groups were compared with vaccine-treated and vaccine- plus cytokine-treated groups of animals. The level of significance was p < 0.05.

Results

Selection, design and synthesis of chimeric HER-2 B cell epitope constructs

We relied on computational methods to predict the B cell epitopes expressed within the ECD of HER-2, as the crystal structure of the protein is not yet available. The basic premise is that the algorithms used in the predictions will always locate regions that are surface-exposed on the protein most likely to be involved in Ab binding. A good correlation exists between the predicted surface-exposed regions of a protein and their antigenicity (46–48). While the individual methods have been applied widely in the past for predicting antigenic determinants in infectious and cellular pro- teins (30, 35, 33), a joint prediction based on a number of different correlates of antigenicity has proved more valuable (29). The ex- tracellular domain was chosen for our analyses, as it is the only part of HER-2 available for binding to Abs induced by a vaccine. Twelve top-ranking epitopes were selected from a total of 144 sequences (10–30 aa long) for detailed studies (Table I). Immuno- nogenicity and antitumor activities of four epitopes (115–136, 410–429, 376–395, and 628–647) have been reported previously (24). To develop a multiepitope vaccine, we selected four high-ranking B cell determinants, sequences 27–45, 316–339, 495–503,
and 605–622 (see Table I for epitope ranking). High ranking sequences are most likely to induce high levels of Abs, although this is not always the case. We excluded the number 2 ranking epitope, 185–216 because it harbors a potential asparagine (N)-linked glycosylation site. We previously found that Abs induced by epitope 115–136, which also presents an N-linked glycosylation site, did not bind to the native protein as a consequence of interference from the sugar moiety (24). In addition, epitope 27–45 was chosen

Table I. Top 12 ranking B cell epitopes identified in HER-2 ECD by computer-aided analyses

<table>
<thead>
<tr>
<th>Predictive Ranking</th>
<th>Position No.</th>
<th>Amino Acid Sequence</th>
<th>Predicted Secondary Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>27–45</td>
<td>TGDWMKLRLPAASPETHLDM</td>
<td>29–34 + 40–45 α-helix</td>
</tr>
<tr>
<td>10</td>
<td>115–136</td>
<td>AVLNLQPVYQTGVGAGSPGG</td>
<td>117–119 turn; 123–129 β sheet</td>
</tr>
<tr>
<td>11</td>
<td>168–184</td>
<td>LWWDFTHNRQNLTTL</td>
<td>168–173 α-helix; 177–180 α-helix; 181–184 β sheet</td>
</tr>
<tr>
<td>2</td>
<td>185–216</td>
<td>DTNRSRACHPCSPMKGSRCWGESEDCQSLT</td>
<td>199–202 turn/loop; 207–210 α-helix; 212–216 β sheet</td>
</tr>
<tr>
<td>5</td>
<td>270–290</td>
<td>ALVYNTDFESNPNPGRT</td>
<td>270–274 β sheet; 277–280 α-helix; 282–284 turn; 287–290 β sheet</td>
</tr>
<tr>
<td>4</td>
<td>316–339</td>
<td>PLINQEVTAEDGKTGAEKCSEKPCA</td>
<td>318–324 α-helix; 329–332 α-helix</td>
</tr>
<tr>
<td>9</td>
<td>376–395</td>
<td>FPESEFDGDGAPNATLPQPE</td>
<td>376–379 α-helix; 382–384 turn; 389–395 α-helix</td>
</tr>
<tr>
<td>12</td>
<td>410–429</td>
<td>LYSIAWPSLPLDSLVSQNLQ</td>
<td>421–429 β sheet</td>
</tr>
<tr>
<td>1</td>
<td>485–503</td>
<td>LFNPQKALLHANTREDE</td>
<td>489–494 α-helix; 499–502 α-helix</td>
</tr>
<tr>
<td>7</td>
<td>560–593</td>
<td>CLPCHPECQPOGTVCTGPEADQCVACAYKDP</td>
<td>571–575 β sheet; 578–581 α-helix; 582–584 β sheet; 585–588 α-helix</td>
</tr>
<tr>
<td>3</td>
<td>605–622</td>
<td>KDLSMPFPKFDPEEGA</td>
<td>607–614 β sheet; 617–620 α-helix</td>
</tr>
<tr>
<td>6</td>
<td>628–647</td>
<td>INTHSCVDLDDKGCPAEQR</td>
<td>626–629 β sheet; 632–634 β sheet; 634–637 α helix; 638–639 turn; 642–646 α-helix</td>
</tr>
</tbody>
</table>

Asparagine (N)-linked glycosylation sites are underlined. Four epitopes (shown in bold) investigated in this study were synthesized collinearly with a promiscuous Th cell epitope, MVF turn (KLLSLIKGVHRELQPSL) at the N terminus. The cysteine to alanine change in the B cell epitope sequence 316–339 is boxed.

Epitopes were ranked as detailed in Materials and Methods.
Secondary structure predictions are based on Chou and Fasman rules (37).
This epitope was tested as part of the multiepitope vaccine based on previously published work (24).

FIGURE 1. Secondary structure of chimeric HER-2 B cell epitope peptides. CD spectroscopy measurements were made in water or 50% TFE. Changes in the spectra indicate the presence of various secondary structural elements, i.e., double minima at 208 and 222 nm is indicative of α-helix, single minimum at 217 nm is indicative of β sheet, and single minimum at 197 nm is indicative of random coil. All peptide spectra show the presence of α-helical structures. The α-helical content calculated based on these CD spectra (see Materials and Methods for details) was 34% in MVF 27–45, 37% in MVF 316–339, 30% in MVF 485–503, and 24% in MVF 605–622.
because it is part of subdomain I (aa 1–160), a unique region of HER-2 that has been speculated to be involved in ligand binding. A subclass of tumor inhibitory HER-2 mAbs that inhibit ligand binding to the cognate receptors interact with subdomain I (49).

Additional changes were made to sequence 316–339, which contains three cysteines at positions 331, 334, and 338. We determined by molecular modeling that residues 334 and 338 form the energetically most stable cysteine-cysteine bond pair (−229.5 kcal/mol) compared with the cysteine bond pairing between residues 331 and 334 (−16.33 kcal/mol) and residues 331 and 338 (−106.5 kcal/mol). Hence, during synthesis we substituted cysteine 331 with alanine to prevent interference with secondary structure formation and aggregation postsynthesis. The molecular modeling results correlate well with experimentally determined disulfide pairings in the ECD of epidermal growth factor receptor (50). The number and the spatial distribution of cysteine residues in the ECD of HER-2 and epidermal growth factor receptor are highly conserved and are virtually identical (51). Each of the HER-2 B cell epitopes was synthesized as a chimera with a promiscuous Th cell epitope (aa 288–302) from MVF at their amino termini. MVF has been previously shown to induce a proliferative response when cultured with lymphocytes obtained from eight human volunteers and six mouse strains (52). These chimeric peptides also incorporate a four-residue linker, GPSL, between the T and B cell determinants, in which glycine and proline in the linker potentiate a β turn in the oligopeptide, whereas serine in that position will favor hydrogen bonds with the free NH of the backbone. Leucine creates a hydrophobic core in the turn. The flexible nature of the linker allows for independent folding of the Th and B cell epitopes (29, 53).

**Secondary structural attributes of chimeric B cell epitope constructs**

Secondary structural elements of the chimeric B cell epitope peptides were evaluated by CD spectroscopy. The wavelength spectra of all the chimeric peptides in trifluoroethanol show a maximum at 190 nm and double minima at 208 and 222 nm (Fig. 1), this pattern is characteristic of α-helical peptides (41). Short peptides (5–50 residues) normally exhibit random coil conformations in water or phosphate buffer. TFE, a solvent that favors internal hydrogen bonding in peptides, is known to stabilize α-helical conformation. TFE is hydrophobic and is thought to impart a plasma membrane-like environment to the peptide similar to the prevailing conditions in vivo, in which the peptide upon binding the MHC class II cleft or the Ag receptor acquires an α-helical secondary structure (54–57). The extent of α-helical content determined experimentally by CD spectroscopy corresponds closely with the theoretical predictions according to Chou and Fasman (37). For example, MVF316–339 shows a mean residue ellipticity of −11,901.4 deg cm²/dmol at 222 nm, which corresponds to an α-helical content of 35.3%. This correlates well with the predicted helical content of 37% for this peptide by Chou and Fasman method (see secondary structure prediction column; Table I). These data imply that the synthetic peptide constructs are approximating the structural features of the native HER-2, therefore suggesting the possibility that the Abs induced by these peptides could cross-react with the cognate protein.

**Immunogenicity of chimeric HER-2 B cell epitopes in outbred rabbits**

The four chimeric HER-2 peptide constructs elicited distinct Ab responses in pairs of immunized outbred rabbits (Fig. 2A). MVF HER-2316–339 was most immunogenic and induced exceptionally high Ab titers of >300,000 just 2 wk after the second booster.

**FIGURE 2.** A, Immunogenicity of chimeric HER-2 peptide constructs in pairs of outbred rabbits. Ab titers were determined against the respective immunogens by ELISA. Each point represents the mean (±SEM) Ab titer of the sera collected from two rabbits. No Abs were detected against the Th cell epitope MVF in sera collected 2 wk after the final immunization (5 ± 2w). Ab titers were defined as the reciprocal of the highest serum dilution with an absorbance of 0.2 or greater after subtracting the background. All data represent the average of duplicate samples. Designation, e.g., 1 ± 3w, on the x-axis represents serum collected 3 wk after the first immunization. B, Levels of peptide Abs that are reactive with HER-2 protein. ELISA plates coated with glycosylated HER-2 ECD recombinant protein (Creative Biomolecules, Philadelphia, PA) were used to determine the levels of native protein-reactive Abs in the antipeptide serum (5 ± 2w bleed). Ab titers are designated by arrows.

MVF HER-2485–503 was moderately immunogenic and elicited highest Ab titers of >30,000 following final immunization. In contrast, relatively low Ab titers (<10,000) were observed against the other two peptide constructs, MVF HER-2316–45 and MVF HER-2485–622 even after the fourth and final boosters. No Abs were detected against the MVF GPSL turn sequence in the HER-2 peptide immune sera (Fig. 2A). In correlation with their immunogenicity, highest levels of anti-HER-2 protein Abs were detected in the 316–339 peptide serum (titer, 16,000), followed by 485–503 and 605–622 (titer, 1000) and 27–45 (titer, 250; see Fig. 2B).

**Cross-reactivity of the peptide Abs with native HER-2**

Synthetic peptides do not often elicit protein-reactive Abs if the structural characteristics of the peptides do not correlate with the native protein. To test whether the Abs induced by the chimeric HER-2 peptides can recognize the native protein, we determined the cross-reactivity of the peptide Abs with the native HER-2 by flow cytometry and immunoprecipitation. In correlation with the Ab titers, tumor cell binding of Abs induced by epitopes 316–339 and 485–503 was twice (mean log fluorescence, 2.32 and 2.18 respectively) as good as the Abs against epitopes 27–45 and
608–622 (mean log fluorescence, 1.53 and 1.47, respectively) in flow cytometric assays (Fig. 3A). With the exception of epitope 27–45, no apparent differences were observed in the levels of the HER-2 protein immunoprecipitated by the other three chimeric peptide-elicited Abs (Fig. 3B). It is possible that epitope 608–622 was better exposed in the HER-2 protein following detergent solubilization of the tumor cells in the immunoprecipitation assay. The lower band in the doublet may be an isoform or an underglycosylated form of HER-2 due to its exceptionally high level of expression in these tumor cells and has also been observed in the past (20, 24, 58, 59).

**Antiproliferative effects of peptide Abs on breast tumor cell lines**

As Abs induced by synthetic peptide vaccines were capable of binding the native receptor, the growth inhibitory effects of peptide Abs were evaluated on two human breast tumor cell lines expressing either very high (BT474) or physiological levels (MCF-7) of HER-2. Abs elicited by peptide epitopes 316–339 and 485–503 inhibited the proliferation of human breast tumor cell line BT474 with minimal effects on the growth of MCF-7 cells (Fig. 4). In correlation with their immunogenicity, the high titrated Abs against the 316–339 epitope were most effective in inhibiting the growth of the BT474 cell line (28%). The relatively lower titrated Abs to the 485–503 epitope inhibited the growth of BT474 cells by 18%. Abs against the HER-2 epitopes 27–45 and 605–622 had minimal effects on the growth of the BT474 tumor cell line. None of the peptide Abs inhibited the growth of the HER-2-negative cell line MCF-7, suggesting the possibility that peptide Abs against epitopes 316–339 and 485–503 selectively inhibit the growth of only HER-2-overexpressing tumor cells. Furthermore, the growth inhibitory effects of the peptide Abs could be completely abrogated by preincubation with the corresponding immunogens (Fig. 4, □).
**FIGURE 5.** Ab responses elicited by multiepitope vaccines in outbred mice. Groups of five outbred mice were vaccinated three times with single- and multiepitope vaccines. ELISA was used to assess the Ab levels against each epitope in the multiepitope vaccine separately as shown on the x-axis. Sera collected 3 wk after the final vaccination (3 + 3w) was used for determination of Ab titers. Bars represent the mean (±SEM) Ab titers of all mice in a group. Ab titers were defined as the reciprocal of the highest serum dilution with an absorbance of 0.2 or greater after subtracting the background. All data represent the average of duplicate samples. Preimmune serum and Th cell epitope (MVF) were negative controls, as indicated in Fig. 2 (data not shown).

**Ab response to multiepitope vaccines**

Combinations of HER-2 mAbs have been shown to mediate synergistic inhibition of tumor growth in both tissue culture and xenografted nude mice (60–63). To mimic such superior tumor growth inhibitory activities with multiple peptide immunogens, we selected three of our best HER-2 B cell epitopes (316–339, 485–503, and 628–647) based on both their immunogenicity and growth inhibitory effects. Groups of five ICR outbred mice were immunized with either single or a combination of two peptide immunogens. The Ab responses to the single immunogens were similar to those observed in outbred rabbits. For the multiepitope immunizations, Ab titers ranged from 10,000 to 100,000 to each of the immunogens in the combination vaccine (Fig. 5). In flow cytometric assays, Abs elicited by the combination vaccines showed no significant differences with single epitope-induced Abs in their binding to HER-2 on the surface of BT474 tumor cells (data not shown).

**Multiepitope peptide vaccine-induced Abs mediate better antitumor effects**

HER-2 mAbs have been shown to inhibit tumor growth by many different mechanisms (64), directly by causing down-modulation of cell surface receptor expression (8, 9) or indirectly by activating effector cells via their FcR (65, 66). To assess the differences in the antitumor activities between single and combination vaccine-induced peptide Abs, we tested both direct and indirect mechanisms of tumor growth inhibition. Treatment of human breast cancer cells (BT474, HER-2<sup>High</sup>) with single and combination vaccine-induced peptide sera lead to a differential decrease in the cell surface expression of the receptor (Fig. 6). While some peptide Abs had no effect on the cell surface expression of HER-2, others caused either low or high levels of receptor down-modulation. Abs elicited by one combination of peptides, 316–339 and 628–647, caused the highest decrease in cell surface expression of the receptor. Indeed, this effect was very similar to that produced by a HER-2 mAb, L26, that was shown to inhibit tumor growth in vivo via receptor down-modulation (9).

Multiple studies have demonstrated the critical role of IFN-γ in the protection against tumor development (67). Vaccines capable of inhibiting tumor growth in wild-type mice were completely ineffective in IFN-γ knockout mice (68). As an index for the indirect activation of immune effector functions via the FcR, we evaluated the ability of peptide Ab-coated tumor cells to activate IFN-γ release from human PBMCs. Two breast cancer cell lines, BT474 (HER-2<sup>High</sup>) and MDA-468 (HER-2<sup>Low</sup>), were incubated for 3 days with various antipeptide sera and human PBMCs. IFN-γ levels secreted into the cell supernatants by the activation of PBMCs by peptide Ab-coated tumor cells were assayed at the end of the 3-day incubation period. Peptide Abs elicited by all combination vaccines activated consistently higher release of IFN-γ compared with Abs elicited by individual epitope vaccines (Fig. 7A). The specificity of the peptide Abs to activate immune cells at the HER-2-expressing tumor site is demonstrated by an almost complete lack of IFN-γ release when cultured with tumor cells lacking HER-2 (MDA-468). As peptide Abs bound to BT474 (HER-2<sup>High</sup>) cells

**FIGURE 6.** Multiepitope vaccine-induced Abs mediate receptor down-modulation. BT474 (HER-2<sup>High</sup>) cells were incubated with preimmune serum (negative control), HER-2 antipeptide sera (1/10 dilution), and a HER-2 mAb, L26 (positive control). After 2 h, tumor cell monolayers were stripped off surface-bound Ab, and the level of HER-2 molecules that remained at the cell surface was determined by binding of 125I-labeled herceptin. The percent receptor down-modulation was calculated as preimmune serum – antipeptide serum/preimmune serum × 100. Each bar represents the mean (±SEM) of two independent experiments.
were capable of activating the release of IFN-γ, we tested whether recombinant IFN-γ can directly inhibit the growth of these cells. We observed a dose-dependent decrease in the number of tumor cells with an increase in the concentration of IFN-γ after 72 h in culture (Fig. 7B). The capacity of recombinant human IFN-γ to inhibit tumor growth directly by binding to the IFN-γ receptors on cancer cells has been observed previously (69–71).

**Vaccination with a combination of chimeric B-cell epitope peptides and IL-12 causes reduction in pulmonary metastases**

We next evaluated the in vivo tumor inhibitory potential of the most effective combination vaccine, 316–339 and 628–647, in conjunction with two cytokines, IL-12 and IFN-α/β, that have been implicated in enhancing the antitumor efficacies of tumor vaccines (15, 72, 73). In some of the earlier studies superior tumor protection correlated with the ability of these cytokines to selectively activate the levels of tumor lysis inducing Abs, predominantly IgG2a (74–76). In addition, both IL-12 and IFN-α/β have been shown to enhance the overall Ab responses to protein Ags (77–82). BALB/c mice were immunized with the two chimeric B cell epitope peptides, MVF 316–339, MVF 628–647, or their combination in conjunction with IL-12 or IFN-α/β. Ab titers were enhanced markedly by IL-12 to MVF 316–339 and MVF 316–339 and MVF 628–647, but not MVF 628–647 (Fig. 8). Interestingly, IL-12 could enhance the Ab titers to MVF 628–647 only when it was administered in combination with MVF 316–339. The Ab levels to both
of the peptides, 316–339 and 628–647, in the combination vaccine were more than doubled when these peptides were administered with IL-12 (Fig. 8). In contrast, IFN-α/β either suppressed or did not alter the Ab response to the peptide immunogens. Enhancement in Ab titers by IL-12 correlated with a selective increase in the levels of IgG2a subclass of Abs (Fig. 9). IL-12 induced a substantial increase in the relative levels of IgG2a combined with a modest suppression of IgG1. In contrast to IL-12, the IgG2a levels were minimally affected by the presence of IFN-α/β (Fig. 9).

Protection from tumor development was evaluated by challenging the vaccinated BALB/c mice with syngeneic tumor cells effectively. These responses were long-lived and lasted >6 mo after the final vaccination in selected patients (27, 28, 83). These clinical trials established proof for the principle that peptide vaccines can be used to boost cross-reactive immunity against HER-2; however, the main obstacle associated with T cell epitope-based peptide vaccines is their applicability in a limited number of individuals as dictated by their MHC haplotype. Given the potential advantage of peptide vaccines to induce cross-reactive immunity against native proteins, it may be possible to develop strategies not only to elicit effective antitumor immunity, but also to circumvent MHC restriction.

To address these issues we have developed a chimeric peptide vaccine approach that incorporates two elements that make it suitable for application in an outbred population: a promiscuous Th cell epitope that activates T cells in the context of many human and mouse MHC haplotypes (52), and a B cell epitope that stimulates Ab responses independent of MHC genetic restriction in contrast to T cell epitope-based peptide vaccines. We have demonstrated in the past that this chimeric peptide vaccine strategy can elicit robust Ab responses in multiple strains of mice and outbred animals (24, 57, 84, 85). In the present study we evaluated four novel HER-2 B cell epitopes to assess the possibility of developing a multiepitope peptide vaccine that could elicit a superior antitumor response. Our hypothesis was that individual B cell epitopes capable of eliciting tumor inhibitory Abs would produce synergistic or additive tumor growth inhibition when administered in combination.

Our studies are the first to report the identification and biological evaluation of B cell epitopes of the HER-2 oncoprotein. While Abs elicited by all four new B cell epitopes cross-reacted with the cognate receptor, only two of these B cell epitopes (316–339 and 485–503) induced tumor inhibitory Abs. Our past (24) and present studies seem to indicate that a combination of two factors, immunogenicity and location of the B cell epitope in the receptor, influences the protective efficacy of the chimeric peptide vaccines. Although epitope 27–45 was thought to be located in the putative ligand-binding region of the receptor, Abs to this epitope did not inhibit tumor growth. The Ab levels to this immunogen and their reactivity with the tumor cells were the lowest (Fig. 3). With the availability of eight peptide Abs targeting specific sites in the extracellular domain, it may be possible in the future to determine the HER-2 subdomains involved in specific functions, such as ligand binding and receptor dimerization.

In addition to the identification of individual epitopes capable of inducing growth inhibitory Abs, we found that vaccination with an appropriate combination of tumor inhibitory epitopes leads to an enhancement of the anti-tumor activities of the peptide Abs. In a study published almost a decade ago, Kasprzyk et al. (61) speculated that a combination of HER-2 mAbs would bring about a nonphysiological multimerization of HER2, in contrast to dimerization induced by individual Abs. The complex lattice so formed can induce superior growth inhibition (61). In support of this speculation, we observed that Abs to a combination of two epitopes mediated much higher levels of receptor down-modulation and release of IFN-γ compared with Abs elicited by single-epitope vaccines. Although Abs elicited by each of the three single-epitope vaccines inhibited tumor cell growth, not all of their combinations elicited Abs with synergistic or additive antitumor activities. This finding implies that formation of a specific receptor lattice is necessary for inducing a desired biological effect. Our studies suggest that Abs targeting HER-2 are capable of inhibiting tumor growth
by both direct and indirect mechanisms, e.g., receptor down-modulation and IFN-γ release. It is possible that Abs trigger many activities at the tumor site, although it is not clear whether these activities are temporally separated or activated in concert. Similarly, HER-2 mAbs, such as herceptin, induce both receptor down-modulation (58) and Ab-dependent cell-mediated cytotoxicity (65) and therefore are thought to inhibit tumor growth by multiple mechanisms (64).

The combinations of peptide vaccines and cytokines were tested for their ability to protect mice from the development of pulmonary metastases. We reasoned that immunization of B cell epitope peptide vaccines in combination with IL-12 or IFN-α/β would produce high titered Abs with elevated levels of IgG2a, which is known to lyse tumor cells most effectively (86, 87). In addition, we speculated that cytokine-activated immune cells would produce tumor inhibitory components (e.g., TNF-α, IFN-γ, and/or NO) in an Ag-dependent or -independent manner and lead to a greater level of protection. IL-12 enhanced the overall Ab titers to the peptide vaccines, which correlated with the capacity of IL-12 to selectively enhance IgG2a levels. In contrast, IFN-α/β altered neither the immunogenicity nor the Ig class switching significantly. Although this result was unexpected, it seems to parallel the findings of studies that evaluated IFN-α/β as a vaccine adjuvant. For example, in chickens, IFN-α/β could enhance Ab responses to tetanus toxoid, but not infectious bursal disease virus, although both these Abs were prepared in the same way by inactivation with formalin (82). This finding suggests that IFN-α/β has distinct effects on different immunogens; therefore, it may be feasible to induce an immune response to peptide vaccines by optimizing the IFN-α/β dose and the immunization scheme.

In correlation with the effects of IL-12 on Ab titers, a statistically significant reduction in the number of tumor metastases was observed with epitope 316–339 and its combination with 628–647 and IL-12. It is unclear why the peptide vaccination did not fully protect the mice from tumor development. Neither peptide Abs nor herceptin could inhibit the growth of RENCA/HER-2 cells when tested in the proliferation assays in the absence of immune effector cells similar to the study described in Fig. 4 (data not shown). It is therefore likely that these tumor cells are not sensitive to direct growth inhibition by Abs. Indeed, the kinetics of tumor formation by both the parental and HER-2-transfected RENCA cells were the same (44), suggesting the possibility that, unlike human tumors, the murine tumor cells do not depend on HER-2 for their survival. It is interesting to note that HER-2 mAbs can inhibit HER-2-dependent human tumor growth in the absence of immune effector cells (88). The direct growth inhibitory effects of these Abs were attributed to their ability to cause receptor degradation (9), block cell division in the G1 phase (89), or inhibit receptor cross-talk attributed to their ability to cause receptor degradation (9), block the murine tumor cells do not depend on HER-2 for their survival.

In correlation with the effects of IL-12 on Ab titers, a statistically significant reduction in the number of tumor metastases was observed with epitope 316–339 and its combination with 628–647 and IL-12. It is unclear why the peptide vaccination did not fully protect the mice from tumor development. Neither peptide Abs nor herceptin could inhibit the growth of RENCA/HER-2 cells when tested in the proliferation assays in the absence of immune effector cells similar to the study described in Fig. 4 (data not shown). It is therefore likely that these tumor cells are not sensitive to direct growth inhibition by Abs. Indeed, the kinetics of tumor formation by both the parental and HER-2-transfected RENCA cells were the same (44), suggesting the possibility that, unlike human tumors, the murine tumor cells do not depend on HER-2 for their survival. It is interesting to note that HER-2 mAbs can inhibit HER-2-dependent human tumor growth in the absence of immune effector cells (88). The direct growth inhibitory effects of these Abs were attributed to their ability to cause receptor degradation (9), block cell division in the G1 phase (89), or inhibit receptor cross-talk (49). Likewise, our peptide Abs were capable of inducing receptor degradation to the levels observed with mAbs (Fig. 6). As RENCA/HER-2 tumor cells do not depend on the presence of HER-2 for their survival, they are less likely to be sensitive to direct growth inhibitory effects of peptide vaccine-induced Abs. This may explain the decrease in the level of protection observed in this murine lung metastases model. These speculations are further supported by the finding that CT26, a chemically induced BALB/c carcinoma cell line transfected with HER-2 similar to the RENCA/HER-2 cell line, was far less sensitive to the growth inhibitory effects of herceptin compared with a herceptin-IL-12 fusion protein in vivo (90). This strategy suggests that activation of additional components of the immune system by IL-12 in an Ag-dependent or -independent fashion can enhance the level of protection. Similarly, the statistically significant reduction in the number of tumor metastases by peptide vaccine plus IL-12 may be partly attributed to the observation that RENCA/HER-2 cells are very sensitive to growth inhibition by recombinant IFN-γ (data not shown), which may have been secreted upon indirect activation of immune cells by peptide Abs in the presence of IL-12, as described in Fig. 7.

A HER-2-dependent tumor model, such as transgenic mice overexpressing HER-2, may be more appropriate than the experimental tumor model used here for assessing the therapeutic potential of vaccines specific to HER-2. Such a model is currently unavailable. Human clinical trials with a combination of peptide epitope vaccines and IL-12 would also provide a better validation for this combination peptide vaccine approach, as both the aggressiveness of human tumor growth and patient survival correlate directly with HER-2 expression levels (2, 3). A National Cancer Institute-supported phase 1b human clinical trial with the multi-epitope vaccine, MVF 316–339 and MVF 628–647, soon to begin here at Ohio State University Medical Center may better predict the applicability of these vaccines for the treatment of HER-2-associated cancers.

The present study identified two novel B cell epitopes capable of inhibiting tumor growth. For the first time a combination of two HER-2 B cell epitopes was shown to induce Abs capable of mediating superior antitumor effects. To our knowledge, IL-12 has not been evaluated with any B cell epitope-based peptide vaccines for tumor therapy.

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