Novel Engineered Trastuzumab Conformational Epitopes Demonstrate In Vitro and In Vivo Antitumor Properties against HER-2/neu


*J Immunol* 2007; 178:7120-7131; doi: 10.4049/jimmunol.178.11.7120
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Novel Engineered Trastuzumab Conformational Epitopes Demonstrate In Vitro and In Vivo Antitumor Properties against HER-2/neu


Trastuzumab is a growth-inhibitory humanized Ab targeting the oncopgenic protein HER-2/neu. Although trastuzumab is approved for treatment of advanced breast cancer, a number of concerns exist with passive immunotherapy. Treatment is expensive and has a limited duration of action, necessitating repeated administrations of the mAb. Active immunotherapy with conformational B cell epitopes affords the possibility of generating an enduring immune response, eliciting protein-reactive high-affinity anti-peptide Abs. The three-dimensional structure of human HER-2 in complex with trastuzumab reveals that the Ag-binding region of HER-2 spans residues 563–626 that comprises an extensive disulfide-bonding pattern. To delineate the binding region of HER-2, we have designed four synthetic peptides with different levels of conformational flexibility. Chimeric peptides incorporating the measles virus fusion “promiscuous” T cell epitope via a four-residue linker sequence were synthesized, purified, and characterized. All conformational peptides were recognized by trastuzumab and prevented the function of trastuzumab inhibiting tumor cell proliferation, with 563–598 and 597–626 showing greater reactivity. All epitopes were immunogenic in FVB/N mice with Abs against 597–626 and 613–626 recognizing HER-2. The 597–626 epitope was immunogenic in outbred rabbits eliciting Abs which recognized HER-2, competed with trastuzumab for the same epitope, inhibited proliferation of HER-2-expressing breast cancer cells in vitro and caused their Ab-dependent cell-mediated cytotoxicity. Moreover, immunization with the 597–626 epitope significantly reduced tumor burden in transgenic BALB-neuT mice. These results suggest the peptide B cell immunogen is appropriate as a vaccine for HER-2-overexpressing cancers because the resulting Abs show analogous biological properties to trastuzumab. The Journal of Immunology, 2007, 178: 7120–7131.

The tumor Ag HER-2 (ErbB2), a member of the epidermal growth factor receptor family, consists of a cysteine-rich extracellular domain (ECD) that has several glycosylation sites, a transmembrane domain, and an intracellular conserved tyrosine kinase domain (1). Lacking a high-affinity ligand, HER-2 functions as a preferential heterodimerization-signaling partner with other members of the EGFR family (EGFR, HER-3, and HER-4) (2, 3) leading to cellular proliferation and differentiation. HER-2 is weakly detectable in epithelial cells of normal tissues (4), but is frequently overexpressed in cancers of the breast, ovary, uterus, lung, and gastrointestinal tract (5–9), HER-2 overexpression in breast cancer patients is associated with a poor prognosis (10). These findings make HER-2 an ideal target for cancer immunotherapy. Numerous Abs directed against the ECD of HER-2 have been generated by immunizing mice with cells expressing HER-2. Dependent upon epitope specificity, the interaction of HER-2 and anti-HER-2 Abs resulted in no effect, inhibition, or stimulation of tumor growth (11). Trastuzumab (Herceptin), a humanized mAb directed against HER-2, has been shown to cause phenotypic changes in tumor cells including down-regulation of the HER-2 receptor, inhibition of tumor cell growth, and reduced vascular endothelial growth factor production (12). In addition, the interaction of trastuzumab with the human immune system via its human IgG1 Fc domain may promote its antitumor properties. In vitro and in vivo studies prove that trastuzumab is very effective in mediating Ab-dependent cell-mediated cytotoxicity (ADCC) against HER-2-overexpressing tumor cell lines (13). Trastuzumab is FDA approved for passive immunotherapy in patients with metastatic HER-2-overexpressing breast cancer (13). In addition, recent studies indicate that 12 mo of trastuzumab treatment along with chemotherapy significantly reduced disease recurrence in patients with early stage breast cancer (14, 15). Trastuzumab has recently been approved in combination with doxorubicin, cyclophosphamide, and paclitaxel in adjuvant treatment for early stage breast cancer after primary therapy.

There are a number of concerns despite the impressive clinical effects of passive trastuzumab application, these include limited duration of action that necessitates repeated treatments at considerable cost. Trastuzumab treatment has been linked with side effects including cardiac dysfunction and congestive heart failure (14–17). The induction of humoral immune responses against HER-2 using active immunotherapy generating a polyclonal, long-lasting immune response has become a desirable objective. To this
end, our laboratory has studied a number of epitopes from the HER-2 ECD identified from computer-aided analysis; we reported the anti-tumor properties of chimeric B cell epitope sequences 628–647 and 316–339 that incorporate a promiscuous T cell epitope (measles virus fusion protein (MVFP)) (18, 19). These studies are the basis for the phase I clinical trial currently being conducted at The Ohio State University James Cancer Hospital.

The crystal structure of the trastuzumab Fab bound to HER-2 reveals that it binds at the C-terminal portion of subdomain IV of the HER-2 amino acids 579–625. Binding of trastuzumab blocks activation of HER-2 by promoting receptor endocytosis as well as blocking proteolytic cleavage of the ECD. Thus, the selection, design, and synthesis of selected regions allow us by active immunization to generate sequence-specific anti-peptide Abs. By examining the mechanistic effects of these Abs such as induction of apoptosis, decreased cell proliferation, Her-2 down-regulation, dephosphorylation, inhibition of signal pathways, inhibition of homo/heterodimerization, ADCC, and complement-dependent cytotoxicity (CDC), we are then able to select these readouts as guides for pursuing in vivo studies as well as for translating these vaccines to the clinic.

In particular, Abs raised against a peptide that could closely mimic the native structure of the pocket-like trastuzumab-binding region of HER-2 are likely to provide more effective functional cross-reactive immune responses with potent anti-tumor properties. We have successfully used several different strategies to mimic the conformation of epitopes in native proteins (21–24). We also demonstrated the use of peptide cyclization by incorporating native disulfide bonds in the 626–649 sequence of HER-2/neu (24) which resulted in improved in vitro and in vivo activity compared with the linear noncyclized peptide.

In this study, we have designed several HER-2 epitopes at the HER-2/trastuzumab interface requiring an elaborate scheme for the successful synthesis, purification, and characterization of these complex epitopes. We show that with varying degrees of reactivity all conformationally restricted peptides were recognized by trastuzumab and blocked the function of trastuzumab inhibiting tumor cell proliferation. All the cyclic and linear epitopes were highly immunogenic in inbred mice eliciting high titer Abs. The 597–626 and 613–626 epitopes elicited strong native-like anti-peptide Abs as evidenced by their reactivity to BT474 and SK-BR-3 breast cancer cell lines using flow cytometric analysis. The 597–626 epitope was immunogenic in outbred rabbits eliciting Abs that recognized HER-2 at the HER-2/trastuzumab interface, inhibited cancer cell growth in vitro, and caused Ab-dependent cell-mediated cytotoxicity. To investigate the efficacy of vaccination in a clinically relevant model, we examined the ability of the conformationally restricted 597–626 epitope as well as its linear counterpart to inhibit the development of mammary tumorigenesis in a transgenic model of HER-2/neu in which the HER-2/neu oncogene is expressed in a tissue-specific manner. We demonstrated that immunization with the 597–626 epitope significantly reduced tumor burden in this aggressive tumor model. We conclude the HER-2 597–626 sequence is a potential vaccine candidate that could be translated to the clinic.

Materials and Methods

Synthesis of linear and disulfide-constrained peptides

HER-2 B cell epitopes 563–598, 585–598, 597–626, and 613–626 were synthesized colinearly with a promiscuous Th epitope derived from the measles virus fusion protein (amino acid 288–302). Peptide synthesis was performed on a Milligen/Biosearch 9600 peptide solid-phase synthesizer using F-moc/t-But chemistry on preloaded clear acid resin (Peptides International) and cleaved using reagent B (trifluoroacetic acid:phenol:water:triisopropylsilane, 90:4:4:2). Three disulfide bonds were introduced in the epitope HER-2 563–598 to more closely mimic the three-dimensional structure of HER-2 protein. Chemoselective protecting groups Cys(Trt), Cys(Acm), Cys(But) were used for desired disulfide pairing. The protecting group from Cys(Trt) comes off in the global cleavage reaction as confirmed by electrospray ionization mass spectroscopy (ESI-MS) analysis. Pure fractions were analyzed using analytical Waters HPLC, pooled together, and lyophilization in 1% acetic acid solution. Clevage of the peptides, native phase-HPLC purification, and lyophilization in acidic medium and prevention of oxidation of free sulfhydryl groups of Cys residues as confirmed by ESI-MS analysis was performed.

Circular dichroism (CD) measurements

Aqueous solutions for CD were prepared by dissolving the freeze-dried peptide in the appropriate amount of water to give a final concentration of 0.5 mM and were used as stock solution for further dilution. CD spectra were recorded on an AVIV model 62A DS CD instrument as reported earlier (24). Mean residue ellipticity (°θM) values were calculated according to the equation: where θ is the recorded ellipticity (deg), M the m.w. of the peptide, n the number of residues in the peptide, c the peptide concentration (milligrams per milliliter), and l the path length of the cuvette. Helicity of peptides was determined according to Chen et al. (25) with reference to mean residue ellipticity of polylysine for 100% α-helix (-35,700 (26).

Animals

Female New Zealand White outbred rabbits and FVB/n inbred mice were purchased from Harlan Breeders. Virgin female BALB-neuT mice (27), BALB/c mice transgenic for the rat-transforming neu oncogene expressed under the control of mouse mammary tumor virus promoter, were bred in our animal facility. Animal care and use was in accordance with institutional guidelines.

Cell lines and Abs

All cell culture medium, FCS, and supplements were purchased from Invitrogen Life Technologies. The human breast tumor cell lines, BT-474, SK-BR-3, and MDA-468, were purchased from American Type Culture Collection and maintained according to the supplier’s guidelines. TUBO cells are a cloned cell line established in vitro from a lobular carcinoma that arose spontaneously in a BALB-neuT mouse (28). TS/A cells are derived from a spontaneous breast cancer of a wild-type BALB/c mouse. HER-2 mAb Ab-2 (clone 9G6) was purchased from NeoMarkers. Rat neu mAb Ab-4 was purchased from Calbiochem. Humanized mouse mAb Herceptin (trastuzumab) was provided by Genentech (South San Francisco, CA).

Immunoassays

To determine the ability of trastuzumab to bind various peptides, a trastuzumab-specific ELISA was performed. Ninety-six-well plates were coated with 100 μl of peptide at 2 μg/ml in PBS overnight at 4°C. Non-specific binding sites were blocked for 1 h with 200 μl of PBS-1% BSA, and plates were washed with PBS-T. Plates were coated with 2 μg/ml of 563–598, SK-BR-3 breast cancer cell lines using flow cytometric analysis. The 597–626 epitope was immunogenic in outbred rabbits eliciting Abs that recognized HER-2 at the HER-2/trastuzumab interface, inhibited cancer cell growth in vitro, and caused Ab-dependent cell-mediated cytotoxicity. To investigate the efficacy of vaccination in a clinically relevant model, we examined the ability of the conformationally restricted 597–626 epitope as well as its linear counterpart to inhibit the development of mammary tumorigenesis in a transgenic model of HER-2/neu in which the HER-2/neu oncogene is expressed in a tissue-specific manner. We demonstrated that immunization with the 597–626 epitope significantly reduced tumor burden in this aggressive tumor model. We conclude the HER-2 597–626 sequence is a potential vaccine candidate that could be translated to the clinic.
buffer). Lysis buffer was composed of 1% Triton X-100, 10% glycercol, 150 mM NaCl, 50 mM HEPES, 1.5 mM MgCl₂, 1 mM EDTA, 10 mM pyro-phosphate, 100 mM NaF, 0.2 mM Na₃VO₄, 10 µg/ml aprotinin, 10 µg/ml leupentin, and 1 mM PMSF. Plates were washed four times with 0.1% Tween/PBS and serial dilutions of rabbit sera (starting at 1/100) were added and the plates were incubated for 2 h on a rocker. Ab binding was detected as described above.

The anti-HER-2 response was measured using a second competitive inhibition assay: plates were coated overnight at 4°C with 100 µl of 500 ng/ml recombinant human HER-2 ECD/Fc chimera (R&D Systems) and plates blocked with 2% BSA for 1 h at 25°C. A constant amount (1/2000 dilution) of rabbit anti-597–626 CYC abs or anti-597–626 NC abs was added to the plates and at the same time various amounts of inhibitor (trastuzumab or isotype control human IgG) was added. Bound anti-peptide rabbit Ab was detected with HRP-conjugated anti-rabbit IgG Ab (mini-

**Peptide immunization and Ab purification**

Mice and rabbits were immunized s.c. at multiple sites with a total of 1 mg rabbits) or 100 µg mice of peptide dissolved in H₂O with 100 µg of a muramyl dipeptide adjuvant, N-acetyl-glucosamine-3-yl-acetyl-t-alal-

**Flow cytometry**

A total of 1 × 10⁶ BT474, SK-BR-3, TUBO, or MDA468 cells were incubated with 1, 10, or 100 µg of mouse or rabbit anti-epitope Abs, HER-2-specific mouse mAb Ab-2 (Lab Vision) and rat neu-specific mAb Ab-4 (Calbiochem) were used as positive controls, and isotypic IgG was used as a negative control. Cells were incubated for 2 h at 4°C in 100 µl of PBS/2% FCS/0.1% Na₃Cytotoxicity was calculated by the formula (percent) lysis = (A – B)(C – B) × 100, where A represents ⁵¹Cr (cpm) from test supernatants, B represents ⁵¹Cr (cpm) from target alone in culture (spontaneous release), and C represents maximum ⁵¹Cr release from target cells lysed with 5% Triton X-100. Results represent the average of triplicate samples.

**Results**

Design and synthesis of conformational and linear peptides

The crystal structure of the extracellular region of HER-2 bound to the trastuzumab Fab reveals that trastuzumab binds subdomain IV of the HER-2 ECD (20). The Ag-Ab interaction is mediated by three regions of HER-2. These regions are composed of loops which consist of residues 579–583 (formed by two disulfide bonds; C563-C576 and C567-C584), 592–595 (cysteine disulfide pairing between C587-C596), and 615–625 (cysteine disulfide bond between C600-C623). We have selected this 64 residue cysteine-rich region of HER-2 for the design of several peptides to minimally mimic the binding epitope. Four constructs (Table 1) encompassing residues 563–626 were designed to contain as least one region of the three binding sequences that make contact with trastuzumab. In addition, we have incorporated the native disulfide bonds in each of these epitopes.

Epitopes containing one or two intramolecular disulfide bond were cyclized using iodine oxidation and characterized as reported (24). In the case of epitope MVF 563–598, which contains three intramolecular disulfide bonds, the first and second disulfide bond formation was performed with in situ reaction using I₂/H₂O, the first disulfide bond formation occurring in the first hour. The addition of water boosts the removal of aceticaminomethyl groups and concurrent formation of second disulfide bond. This was confirmed by polyethyleneoxide (PEO)-maleimide reaction; biotinylation agent PEO-maleimide, which attacks free sulphydryl groups to form addition product and therefore can be used to determine the completion of disulfide pairing and was confirmed by ESI-MS (24). Two Cys(Bu₃) groups remained intact during the cyclization process.
procedure. The third disulfide bond was formed by silyl-chloride-sulfoxide method and the completion of third disulfide bond was confirmed by PEO-maleimide reaction; no PEO-maleimide addition was observed, which was confirmed by ESI-MS characterization (Fig. 1). Linear peptide was generated by DTT reduction. These spectroscopic results indicate that we have successfully synthesized conformational peptides incorporating three disulfide bonds.

The HER-2 B cell epitopes were synthesized colinearly with a promiscuous Th epitope derived from the measles virus fusion protein (MVF) (amino acids 288–302). A four-residue linker sequence, GPSL, connects the Th and B cell epitopes; this linker sequence forms a hairpin loop. The small, flexible nature of glycine and the ability of proline to readily form the cis-conformer make these amino acids amenable to tight turns. Serine favors hydrogen bonds with the free amide of the backbone and interacts favorably with solvent and leucine is buried in the hydrophobic core. Independent folding of the Th and B cell epitopes is achieved using the flexible linker sequence (30, 31).

Specificity of peptides binding to trastuzumab
To examine the ability of trastuzumab to bind the conformational cyclized synthetic peptides, a trastuzumab specificity ELISA was developed. Polystyrene plates were coated overnight with various peptides and the following day probed with trastuzumab. Trastuzumab binds to all four of the peptides MVF 563–598, 585–598, 597–626, and 613–626 but not the irrelevant control peptide MVF 316–339 (Fig. 2A). The 563–598 peptide exhibited the highest reactivity with the mAb. Moreover, trastuzumab binds the synthetic peptide MVF 563–598 that incorporates the natural disulfide pairings of HER-2 in a dose-dependent manner and to a lesser extent the linear noncyclized peptide (Fig. 2B). These results suggest that the conformation induced by cyclization mimics the native sequence better than the flexible peptide.

Conformational peptides prevent trastuzumab inhibiting tumor cell growth
The ability of trastuzumab to bind the ECD of HER-2 and inhibit the downstream signaling of HER-2, resulting in growth inhibition of HER-2-overexpressing cell lines has been demonstrated extensively (13). Because we have shown that trastuzumab recognizes all four of the peptides, we investigated whether these peptide prevent trastuzumab from inhibiting tumor cell growth. By preincubating trastuzumab with peptide, the response rate of the breast cancer cell line BT474 to trastuzumab was blocked by all peptides (MVF 563–598CYC, MVF 585–598CYC, MVF 597–626CYC, and MVF 613–626) in a dose-dependent manner, while an equivalent concentration

![FIGURE 1. Synthetic strategy for generating three disulfide pairings in peptide MVFGPLS563–598 from the trastuzumab-binding region of HER-2. Disulfide bonds are selectively introduced in the epitope; the peptide was synthesized with chemoselective protected cysteine residues. Each cysteine pair of a disulfide bond was identically protected to achieve desired intramolecular cyclization. The PEO-maleimide reaction and subsequent ESI-MS characterization confirmed the intramolecular disulfide pairing.](http://www.jimmunol.org/)

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**Table I. Peptides synthesized from the trastuzumab-binding region of HER-2**

<table>
<thead>
<tr>
<th>Designation</th>
<th>Peptide Sequence</th>
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<tbody>
<tr>
<td>563 CYC</td>
<td>CHPECQPQVFCTCPGPDQCVACAHYKDPFPCA-VCAHYSQGPPCPVCOOH</td>
</tr>
<tr>
<td>585 CYC</td>
<td>VACAHYKDPFPCA-VCAHYSQGPPCPVCOOH</td>
</tr>
<tr>
<td>597 CYC</td>
<td>VARCPGVKPDLYMPICVFPDEEGACQP</td>
</tr>
<tr>
<td>613</td>
<td><a href="http://www.jimmunol.org/">underlined amino acids were mutated from Cys to Leu so as not to interfere with natural disulfide formation.</a></td>
</tr>
</tbody>
</table>

* Peptides containing disulfide bonds (CYC) are shown; linear versions (NC) were also synthesized (not shown in table).
* Residues involved in binding trastuzumab are shown in bold; a possible N-linked glycosylation site in the 563–598 epitope is boxed. Underlined amino acids were mutated from Cys to Leu so as not to interfere with natural disulfide formation.

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7123The Journal of Immunology

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of control peptide had no effect (Fig. 3). At a concentration of 60 μg/ml only the 563–598 and 597–626 peptides had a statistically significant decrease (p < 0.05) in inhibition compared with nonspecific peptide (MVF 127–144), indicating that both MVF 563–598CYC and MVF 597–626CYC peptides bind to trastuzumab and prevent the mAb from inhibiting tumor cell growth.

Immune response of peptide constructs in FVB/n mice

We investigated the immune response of each of these constructs in FVB/N mice (n = 5–10). Each of the constructs elicited high-titered Abs in mice, as evidenced by Ab titers over 100,000 (Fig. 4A). Notably both the cyclized (CYC) and linear (NC) forms of the 563–598 epitope were most immunogenic and induced titers greater than 100,000 in each mouse 2 wk after the second booster injection. In addition, mice receiving the cyclized versions of 563–598 and 597–626 showed a greater immune response compared with mice receiving the linear versions of the peptides. IgG1, IgG2a, and IgG2b were the major isotypes in the mouse sera elicited by the various peptide constructs (Fig. 4B). Interestingly, sera against peptide constructs MVF 585–598CYC, MVF 597–626CYC, and MVF 597–626NC generated the largest proportion of IgG2a (29–32% of total IgG), an isotype associated with an effective antitumor response (28, 32, 33).

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

It is essential for Abs raised against a synthetic peptide to recognize the native protein to be considered a potential vaccine candidate. We tested the binding of FVB/N purified Abs to the HER-2-overexpressing human breast cancer lines BT474 and SK-BR-3 by immunofluorescence staining of a single-cell suspension (Fig. 5, left and middle panel). Abs generated against 597–626 bound well within 1 log of HER-2-specific mouse mAb Ab-2 in both BT474 and SK-BR-3 (Fig. 5C) cell lines and showed the largest shift of the anti-peptide Abs. Abs against the 613–626 epitope were capable of recognizing the native protein (Fig. 5D). However, Abs to 563–598 and 585–598 showed weak binding to both cell lines (Fig. 5, A and B).

Anti-peptide Abs did not demonstrate binding to MDA468 (Fig. 5, right panel), a non-HER-2-overexpressing breast cancer cell line. The 563–598 sequence harbors a putative N-linked glycosylation site at residue 571 (Table I, boxed residues). The published crystal structure of HER-2 bound to trastuzumab was enzymatically deglycosylated, whereas the crystal structure of HER-2 bound to the pertuzumab Fab reveals a sugar moiety at position 571 (34). Abs against 563–598 peptides did not cross-react with SK-BR-3 cells treated with tunicamycin, which prevents addition of N-linked oligosaccharides to proteins (data not shown). The lack of recognition of Abs elicited to 563–598 may be due to conformational differences when HER-2 is glycosylated. The 585–598 epitope is most likely too short (containing only 14 aa) to elicit cross-reactive Abs specific to HER-2.

Effects of 597–626 peptide constructs in outbred rabbits

Based on the ability of Abs induced by the synthetic peptide vaccine 597–626 to bind the native receptor with high specificity and to be recognized by trastuzumab, this epitope was considered to be the most promising vaccine candidate. We therefore evaluated the immunogenicity of the 597–626 construct in outbred New Zealand White rabbits to generate a large quantity of Abs for in vitro studies. Both the cyclized and linear peptides elicited high-titered Abs (Fig. 6A).

IgG was the predominant isotype generated in rabbits. Abs elicited by 597–626 CYC construct contained 95.8% IgG, 3.7% IgM, and 0.5% IgA, whereas Abs elicited against 597–626NC had 94.4% IgG, 5.0% IgM, and 0.6% IgA (data not shown). We examined the cross-reactivity of the rabbit Abs to the native protein using flow cytometry. Abs raised against both 597–626CYC and NC recognized both HER-2-overexpressing cell lines BT474 (Fig.
6B) and SK-BR-3 (Fig. 6C) but MDA468 cells (Fig. 6D) which do not overexpress HER-2. This data suggest that the Abs induced by the vaccine constructs were specific for the HER-2 protein. In addition, the binding of anti-peptide Abs to HER-2 was measured using two ELISA. First, we performed a sandwich ELISA in which SK-BR-3 cell lysate was used as a source of HER-2. Fig. 7A reveals that anti-597–626 Abs (1/100 dilution) recognize HER-2 in a similar manner as trastuzumab (20 μg/ml). Next, the ability of 597–626 anti-peptide Abs to bind recombinant human HER-2/Fc chimera was examined. Both 597–626CYC and 597–626NC Abs had a titer of 8000 (Fig. 7B) against the native protein. To test whether the 597–626 anti-peptide Abs bound to the same epitope as trastuzumab, the mAb or isotype control human IgG were used as competitor for Ag binding in ELISA experiments. Anti-peptide Abs were allowed to bind to immobilized HER-2 in the presence and absence of various concentrations of trastuzumab or human IgG. At a concentration of 1000 ng/ml, trastuzumab was able to inhibit the binding of anti-597–626CYC Abs (Fig. 7C) and anti-597–626NC Abs (Fig. 7D) to HER-2 by 75.0 and 70.9%, respectively. The results demonstrate that both anti-597–626CYC and anti-597–626NC Abs recognize the same or similar determinant as that of trastuzumab.

FIGURE 4. Immunogenicity of HER-2 peptide constructs in FVB/N mice. A. Indirect ELISAs were performed; results of individual mice are shown (n = 5–10). Ab titers were defined as the reciprocal of the highest serum dilution with an absorbance of 0.2 or greater after subtracting the background. 1y+3 indicates the titer of blood drawn 3 wk after the first immunization. Preimmune sera was used as a negative control (data not shown). B. Two weeks after the final immunization (3Y+2), the level of Ig subtypes were measured using a mouse isotyping kit. The concentrations of IgG3, IgA, and IgM were <10% (data not shown).

FIGURE 5. Cross-reactivity of peptide Abs to breast cancer cell lines. The reactivity of purified Abs from immunized FVB/n mouse sera was tested with BT474 (left panel), SK-BR-3 (middle panel), which are breast cancer cell lines that overexpress HER-2, and MDA468 (right panel), a non-HER-2 overexpressing cell line, using flow cytometric analysis. Abs shown were raised against peptides (A) 563–598, (B) 585–598, (C) 597–626, and (D) 613–626. Ab binding was detected with goat-anti-mouse FITC-conjugated secondary Abs. Histograms indicate linear peptide Abs (light gray shading), cyclized peptide Abs (dark gray shading), normal mouse IgG (negative control, light gray line histogram), and Ab-2 (positive control, black shading).
Effect on breast cancer cell viability and proliferation

We next examined the effect of 597–626 peptide Abs on tumor cell survival in vitro (29). BT474 cells were plated overnight; the next day cells were treated with either 20 μg/ml Abs elicited from 597CYC, 597NC, trastuzumab, normal rabbit IgG, or medium alone. Cell viability was measured after 72 h by trypan blue exclusion. Cells treated with Abs elicited from 597–626CYC and 597–626NC had 61 and 46% viability, respectively, compared with untreated cells (Fig. 8A) whereas trastuzumab treated cells had 21% viability, indicating that Abs against both conformational
and linear form of 597–626 are able to decrease BT474 cell viability. We then examined the ability of anti-peptide Abs to effect in vitro tumor cell proliferation using the MTT assay. As shown in Fig. 8B, Abs elicited by peptide epitopes 597CYC and 597NC had a similar effect on the proliferation of BT474 cells (19 and 18% inhibition, respectively), whereas trastuzumab demonstrated a 59% inhibition on tumor cell proliferation. These findings demonstrate that Abs against both conformational and linear forms of 597–626 are able to diminish cell viability as well as have antiproliferative effects on BT474 cells in vitro.

**Ability of anti-peptide Abs to mediate ADCC**

It has been well-documented that in vivo the Fc portions of Abs can be of foremost importance for efficacy against tumor targets (35). When Fc binding is reduced or completely removed, trastuzumab loses virtually all of its antitumor activity in vivo (36). Consequently, Fc-dependent ADCC is critical for in vivo efficacy. Therefore, we measured the ability of the anti-peptide Abs to mediate ADCC in vitro. Peptide Abs elicited in rabbits against both the cyclized and linear peptide invoked lysis of the breast cancer cell line BT474 in the presence of human PBMCs, analogous to trastuzumab (Fig. 9). These results suggest that Abs raised against both conformational and linear form of 597–626 are able to mediate ADCC in a similar manner as trastuzumab.

**Effect of conformational restriction on the MVF 597–626 sequence**

The chimeric peptide epitopes are quite complex involving several turns, antiparallel β-sheets, β-turn, and some helical structure in the MVF epitope. Our structural analysis of the cyclized and non-cyclized peptides by CD (data not shown) was quite similar involving those secondary elements. Peptide MVF 597–626CYC is partially folded because of conformational constraints imposed by one disulfide bond. This disulfide bond reduces the conformation freedom of the polypeptide chain. CD measurements (data not shown) at 100 μM concentration shows minimum at 198 nm as well as 195 nm indicating population of turns in this peptide. The peptide MVF 597–626NC shows only one minimum at 198 nm indicating different topology of...
A rat neu; the human 597–626 sequence has 93% homology with the
Animals rapidly develop tumors; in preliminary studies using un-
mainly with glutamic acid favors HER-2/neu
examined whether Abs raised against 597–626 were capable of
determine the immunogenicity of the 597CYC construct in BALB-neuT
were represented by an individual box. Ab titers were
dimensions: 594.0x792.0
The data are presented as the average tumor size per group
were determined by the formula (long measurement
of isolated
significant prevention of tumor growth compared with the naive group or the
immunization2)/2. The data are presented as the average tumor size per group
of disease-free survival in vaccinated
A, ELISAs were performed to
did not require constraining by disulfide bonds as they are con-
the protein as well as the individual peptide may fold independently
were performed twice a week on each of 10 mammary glands. Tumor
of blood drawn 3 wk after the first immunization.

Effects of peptide constructs in BALB-neuT mice
We used the BALB-neuT transgenic mouse mammary cancer model as a measure of the ability of the peptide constructs to re-
reduce tumor progression. BALB/c inbred mice transgenic for the
transformation activated rat HER-2/neu oncogene under the control
of a mammary-specific promoter is likely the most aggressive
model of HER-2/neu carcinogenesis (27). A point mutation that
replaces the valine residue at position 664 in the transmembrane do-
main with glutamic acid favors HER-2/neu homo- and heterodimer-
ization and renders the neu gene product constitutively active (37).
Animals rapidly develop tumors; in preliminary studies using un-
treated mice, all animals developed tumors by 25 wk of age.
There is 88% sequence homology between human HER-2 and
rat neu; the human 597–626 sequence has 93% homology with the
rat neu sequence, with two disparate amino acids (Fig. 10A). We
examined whether Abs raised against 597–626 were capable of
recognizing the rat neu receptor using the TUBO cell line, a cell
line established in vitro from a lobular carcinoma that arose sponta-
nously in a BALB-neuT mouse. As depicted in Fig. 10B, Abs against both cyclized and linear forms of 597–626 were shifted
relative to normal rabbit IgG and were comparable to Ab-4, a
mouse mAb that binds rat neu. Flow cytometric analysis of the
non-neu-expressing TS/A cell line demonstrated that no Abs
bound this cell line (Fig. 10C).

Based on these results, the conformationally restricted MVF 597–626 was chosen for study in female transgenic BALB-neuT
mice. Previous studies have shown the age at which mice are im-
munized is critical (33). Transgenic mice that began receiving den-
dritic cells transduced with adenovirus expressing the neu oncop-
rotein past 6-wk old had larger tumor burden compared with mice
receiving the vaccine at 5–6 wk of age (38). Transgenic mice were
immunized with 597–626CYC beginning at 5–6 wk of age. Mice
received two booster immunizations at 3-wk intervals and subse-
sequently two additional immunizations at 4-wk intervals. Impres-
sively, 597CYC elicited high-titer anti-neu Ab responses in all
mice 3 wk after the third immunization (Fig. 11A). At 25 wk of
age, untreated mice and mice immunized with irrelevant peptide
had an average tumor burden of 3486 mm³ (±1166) and 2720
mm³ (±1163), respectively (p = 0.6441). Mice immunized with
597–626CYC had a significant reduction in tumor burden (p <
0.0001), with an average tumor burden of 378 mm³ (±228.0) (Fig.
11B). The in vivo antitumor activity observed here correlates with the
in vitro studies indicating the 597–626 construct is capable of
inhibiting tumor cell growth as well as mediate ADC in a
similar manner as trastuzumab. These results indicate that the
conformationally restricted 597–626 epitope is capable of sig-
nificantly inhibiting tumor growth in a mouse model that par-
allels several characteristics of the stepwise mammary carcino-
genesis in women.

Discussion
HER-2 vaccines have been designed that use whole cells expressing
tumor Ags (39–41), proteins (42), as well as DNA expression
plasmids (43–45). Most of the immunotherapies targeting the
HER-2 oncoprotein have focused on T cell epitopes, and several
studies have produced notable results indicating that vaccinated
patients can develop immunity to HER-2 peptides and native pro-
tein (46–48). Recent clinical trial results using a HER-2 CTL
epitope showed an increase in disease-free survival in vaccinated
HER-2/neu-expressing breast cancer patients (85.7%) compared
with the control group (59.8%) (49). However, the effectiveness of
a CTL vaccine for clinical use is limited to patients who express
the appropriate HLA haplotype. To date mAbs, based on B cell
immune responses, and not vaccines to activate the T cell immune
responses, have been successful in clinical trials and approved for
usage (50). In particular, the clinical efficacy of trastuzumab sug-
ests that the generation of a robust and focused humoral immune
response may be biologically significant for tumor defense.
Before the publication of the three-dimensional structure of
HER-2 (20), the identification of HER-2 B cell epitopes was
achieved through computer-aided analysis (18, 19, 51). In addi-
tion, several HER-2 B cell mimotopes have been identified through
phage display (11, 52, 53). Riemer et al. (52) used a constrained
10-mer random peptide phage display library to identify peptide
mimotopes to trastuzumab; Abs raised against one of these pep-
tides recognized HER-2/neu and caused internalization of the re-
ceptor from the cell surface in a similar manner as trastuzumab
(52). Although this peptide sequence bears no sequence homology
to HER-2, it was matched to the third loop of HER-2 at the HER-
2/trastuzumab interface using computational methods (54). Jiang
et al. (53) identified another mimotope that matched to an epitope between loops 1 and 2 of HER-2 at the HER-2/trastuzumab interface. These studies indicate that all three loops are important for trastuzumab-binding HER-2.

We have designed four peptide constructs that each contains at least one of the three loops of HER-2 involved in binding trastuzumab. The use of synthetic peptides to represent protein domains is restricted by conformational issues. The protein fragment of interest is stabilized by secondary and tertiary interactions in the native protein, but the matching peptide in solution will typically have a random coil structure. Lacking structural restraints, the flexibility of peptides can lead to varied conformations presented to the immune system, most of which are non-native (55). Linear peptides are highly flexible and can adopt a variety of conformations in solution. However, only a few of these conformations are responsible for their immunoreactivity (56). One approach to achieve molecular mimicry to the parent protein is through constricting the peptide by cyclization if the natural sequence bears cysteine residues that are paired to provide loop sequences with enhanced stability. Cyclic peptides can cause preferred spatial arrangements that duplicate the bioactive conformation, resulting in improved binding and immunological properties.

In our previous studies, we demonstrated that conformational cyclic epitopes HER-2 sequence 626–649 (24) had the desired secondary structural characteristics as determined by CD measurements. Abs against the cyclized epitope bound the HER-2 protein with a higher affinity than the noncyclized epitope and were twice as effective in ADCC assay and in reducing tumor growth in transgenic mice. However, in the present study, we were unable to differentiate between the conformations of the epitope region spanning residues 597–626. Similarly, the Abs that were generated were also very similar in reactivity and biological efficacy. We have designed peptide constructs to include the native disulfide bonds of the epitopes to more closely mimic the native structure. Each of the peptide constructs were recognized by trastuzumab, with 563–598 showing the greatest recognition. In addition, all peptides were able to prevent trastuzumab from inhibiting tumor cell growth. However, Abs raised against the 563–598 epitope do not, to an appreciable extent, recognize HER-2 as measured by flow cytometry. This may be due to the asparagine-linked glycosylation site (571-NGS) found within this sequence. It has been reported previously that trastuzumab’s mouse counterpart mAb, 4D5 recognized glycosylated HER-2 and unglycosylated HER-2, indicating that either the mAb recognizes a conformation of the protein attained only when it is glycosylated, or, conversely, the epitope recognized by 4D5 comprises partly of carbohydrate (57). Abs against the 563–598 peptide inability to bind native HER-2 may due to a local conformational change when HER-2 is glycosylated, or, alternatively, dominant epitopes in the 563–598 peptide are not surface exposed on the native protein. However, Abs raised against the third loop of HER-2 (597–626) did recognize HER-2 and were investigated further. The 597–626 epitope was immunogenic in outbred rabbits; these polyclonal Abs recognized HER-2. In addition, competition experiments revealed that trastuzumab was able to inhibit the binding of anti-597 Abs to HER-2, indicating the anti-peptide Abs bound the same epitope as trastuzumab.

Trastuzumab is known to affect tumor growth by both direct and indirect mechanisms. The direct mechanisms involve binding to HER-2 and altering the receptor’s signaling properties that can result in tumor growth cell inhibition (13). Anti-597 Abs were able to diminish cell viability as well as inhibit tumor cell growth of the BT474 cell line in a similar manner as trastuzumab. The indirect mechanisms involve the classical pathways in which trastuzumab kills tumor cells by mediating ADCC and CDC. We show here that anti-597 Abs were able to mediate ADCC in a manner similar to trastuzumab. Although the mechanisms by which Abs exert their therapeutic effects are still being debated, the putative mechanisms are either direct (i.e., block signaling functions, internalization of receptors, reduce proteolytic cleavage of receptors) or indirect action mediated by the immune system CDC, ADCC. Thus, we have attributed the antitumor response and protective efficacy to the generation of anti-peptide Abs and not to a T cell-mediated immune response. However, because we did not evaluate the types of tumor infiltrating leukocytes that may have been induced after peptide vaccination or in mammary tumors of BALB-neuT mice after defined stages, we cannot discount the possibility that a T cell response (CD8+ or CD4+) to the vaccine may have played a role in efficacy. In future studies, we will explore the tumor microenvironment and its interface with the tumor cells because it is known that some tumors are infiltrated with lymphocytes, macrophages, and granulocytes.

To demonstrate in vivo efficacy of the 597–626 vaccine, we used the BALB-neuT transgenic mouse model. Although a more desirable model would be mice transgenic for human HER-2, only recently has a model been described in which animals form tumors (58). Abs against the 597–626 epitope were cross-reactive with rat neu protein, thus we used this animal model. It has been shown that the induction of anti-Her-2/neu Abs are both necessary and sufficient for protection of BALB-neuT mice from developing tumors as shown by depletion of CD4+ and CD8+ cells (32, 33). Thus, this animal model is advantageous to studies that identify B cell epitopes necessary in the protection of BALB-neuT transgenic mice from developing tumors. By 25 wk of age, mice immunized with the 597CYC construct had a statistically significant reduction in tumor burden compared with both naive and MVF-immunized mice. Because we have demonstrated that Abs against the 597–626 epitope bind to HER-2 at the trastuzumab interface, the mechanism of action of the endogenous tumor protective in BALB-neuT mice most likely include down modulation of the HER-2/neu receptor as well as interaction with the immune system via the Fc domain of endogenous Abs against the 597–626 epitope.

In summary, we report here an epitope that mimic the HER-2/trastuzumab interface capable of inducing Abs with antitumor properties that significantly reduces tumor burden in vivo in transgenic mice. There are inherent limitations of passive immunotherapy with trastuzumab including unequal tissue distribution, limited half-life, prolonged administration, possible immunogenicity with high dosages, and cardiotoxicity. Immunotherapy with peptide vaccines that produces endogenous Abs may be more valuable than repeated administration of an exogenous mAb. Peptide vaccines are easy to produce, amenable to quality control, and cost effective. The active generation of Abs with similar characteristics as trastuzumab has the potential to suppress the development of HER-2-overexpressing breast cancers.

Disclosures
The authors have no financial conflict of interest.

References


