

Generation of Peptide Mimics of the Epitope Recognized by Trastuzumab on the Oncogenic Protein Her-2/neu¹

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Immunizations with the oncogenic protein Her-2/neu elicit Abs exerting diverse biological effects—depending on epitope specificity, tumor growth may be inhibited or enhanced. Trastuzumab (herceptin) is a growth-inhibitory humanized monoclonal anti-Her-2/neu Ab, currently used for passive immunotherapy in the treatment of breast cancer. However, Ab therapies are expensive and have to be repeatedly administered for long periods of time. In contrast, active immunizations produce ongoing immune responses. Therefore, the study aims to generate peptide mimics of the epitope recognized by trastuzumab for vaccine formulation, ensuring the subsequent induction of tumor growth inhibitory Abs. We used the phage display technique to generate epitope mimics, mimotopes, complementing the screening Ab trastuzumab. Five candidate mimotopes were isolated from a constrained 10 mer library. These peptides were specifically recognized by trastuzumab, and showed distinctive mimicry with Her-2/neu in two experimental setups. Subsequently, immunogenicity of a selected mimotope was examined in BALB/c mice. Immunizations with a synthetic mimotope conjugated to tetanus toxoid resulted in Abs recognizing Her-2/neu in a blotted cell lysate as well as on the SK-BR-3 cell surface. Analogous to trastuzumab, the induced Abs caused internalization of the receptor from the cell surface to endosomal vesicles. These results indicate that the selected mimotopes are suitable for formulation of a breast cancer vaccine because the resulting Abs show similar biological features as trastuzumab. *The Journal of Immunology*, 2004, 173: 394–401.

The oncogenic protein Her-2/neu (erbB-2), a member of the family of epidermal growth factor (EGF)³ receptors, functions via triggering intracellular tyrosine kinase-dependent processes (1). Her-2/neu is modestly expressed in normal adult tissues (2), where it regulates cell growth and differentiation (3). It is overexpressed in ~30% of invasive breast cancers and ~70% of ductal carcinomata in situ (4, 5), in a small percentage of melanomas (6), and in other malignancies originating from various organs, including ovary (4), kidney, colon (7), and bladder (8). Overexpression of Her-2/neu leads to increased responsiveness to stromal growth factors of the EGF family, resulting in potentiated signaling, which ultimately results in malignant proliferation (9). As Her-2/neu contains a large extracellular domain of 110 kDa, it has attracted interest as a target for immunological attack. This view was further strengthened by the discovery of humoral (10) and cellular immune responses against Her-2/neu in some patients with Her-2/neu overexpressing tumors (11). Moreover, Her-2/neu peptide-specific T cells could be isolated from peripheral blood of

cancer patients as well as of healthy individuals (12), indicating that tolerance to this self-Ag is not absolute.

As a series of mAbs directed against the Her-2/neu receptor became available and their interaction with Her-2/neu overexpressing cells was characterized, differences between their intrinsic actions were demonstrated: depending on epitope specificity, the interaction of Her-2/neu and anti-Her-2/neu Abs was shown to result in either inhibition or enhancement of tumor proliferation (13–16). This emphasizes that an inappropriately induced immune response could result in detrimental effects. In contrast, a selective immune response toward “inhibitory” epitopes may lead to cancer cell death (17).

In this setting, the phage display technique is an attractive tool to generate mimics of an epitope recognized by a given tumor growth-inhibiting Ab (18). For this purpose, we chose trastuzumab (herceptin), a growth-inhibitory humanized monoclonal anti-Her-2/neu Ab, approved by the U.S. Food and Drug Administration for passive immunotherapy in the treatment of patients with advanced Her-2/neu overexpressing breast cancer. The Ab binds to the extracellular domain of Her-2/neu and inhibits the downstream signaling cascade, resulting in growth inhibition of Her-2/neu overexpressing tumor cells (17). This inhibitory capacity was found to be associated with internalization of the receptor-Ab complex and movement into endocytic vesicles (3, 15, 19). Trastuzumab proved to be successful as monotherapy of pretreated patients (20, 21), monotherapy as first-line treatment of metastatic disease (22), and in combination with various cytotoxic agents including paclitaxel, docetaxel, gemcitabine, vinorelbine, platinum compounds and other drugs (23–27). Building upon these described impressive clinical effects of passive trastuzumab application, we aimed to generate vaccine constructs for active immunization, with the potential to elicit in vivo production of Abs with immunological and biological properties equivalent to trastuzumab.

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³ Abbreviations used in this paper: EGF, epidermal growth factor; TT, tetanus toxoid.

Materials and Methods

Cell lines, membrane fraction cell extracts, and total cell lysates

The Her-2/neu positive human mammary carcinoma cell line SK-BR-3 (HTB-30; American Type Culture Collection (ATCC), Manassas, VA) was grown in McCoy's medium (Life Technologies, Inchinnan, U.K.) supplemented with 10% FCS, 1% glutamine, 1% penicillin/streptomycin, and 50 μ g/ml gentamicin sulfate. The human mammary cell line MDA-MB-468 (HTB-132; ATCC), which is Her-2/neu-negative, was grown in Leibovitz-15 medium (Life Technologies) and supplemented as previously described.

Membrane fraction cell extracts were prepared as previously described (28). Total cell lysates were made (outlined in Ref. 29) using a modified lysis buffer, containing 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and a protease inhibitor mixture (Complete; Roche, Basel, Switzerland), at pH 7.5. The protein concentration was determined photometrically, using bicinchoninic acid (BCA Protein Assay kit; Pierce, Rockford, IL). Extracts were aliquoted and stored at -80°C .

Monoclonal Abs

Trastuzumab (herceptin), a humanized IgG1 mAb, was purchased from Roche (Hertfordshire, U.K.). Rituximab (rituxan), also a humanized IgG1 mAb and directed against CD20, was purchased from Genentech (IDEC Pharmaceuticals, San Diego, CA) and used as an isotype control.

Phage library and biopanning

Three successive rounds of panning with trastuzumab were performed with a random phage library (CL10), expressing cysteine-flanked decapeptides, circularized by disulfide bridging, fused to pIII of the filamentous phage M13 (30). Biopannings were performed (outlined in Ref. 31) with some modifications. In short, ELISA plates (Nunc, Roskilde, Denmark) were coated with 40 μ g/ml trastuzumab in bicarbonate buffer, pH 8.5. Wells were blocked with PBS/1% dry milk and incubated with an aliquot of the phage library ($\sim 5 \times 10^{10}$ phage particles) in PBS/1% dry milk/0.1% Tween 20, at room temperature for 2 h. Unbound phage particles were removed by extensive washing with PBS/0.5% Tween 20. Bound phage was eluted with 0.1 M glycine, pH 2.2, and immediately neutralized. Phage was amplified in *Escherichia coli* K91, precipitated from the bacterial culture supernatant with polyethylene glycol, and either immediately used for the next round of biopanning or stored at -20°C . For titer determination, aliquots of the eluate or the amplificate were plated in serial dilutions on Luria broth agar plates containing 75 μ g/ml kanamycin.

Colony screening assay

After each round of biopanning, colony screenings for selection of specific phage clones were done according to Barbas (32). Immunoscreeenings were performed with trastuzumab and isotype control rituximab (2.5 μ g/ml in PBS/casein). Bound Ab was detected by ^{125}I -labeled anti-human IgG (IBL, Hamburg, Germany). Filters were washed, dried, and exposed to Biomax-MS films (Kodak, Rochester, NY) at -70°C . Positive clones were amplified as previously described.

DNA sequencing

Single strand phage DNA was prepared using a QiaPrep Spin M13 kit (Qiagen, Hilden, Germany). The amount of prepared DNA was checked with an EtBr₂/0.7% agarose gel under UV illumination. DNA sequencing was done by IBL (Vienna, Austria).

Specificity ELISA

ELISA plates (Nunc) were coated with trastuzumab or rituximab, 10 μ g/ml in PBS by overnight incubation at 4°C . Plates were then washed with PBS/0.1% Tween 20, and nonspecific binding was blocked by incubation with PBS/0.1% Tween 20/1% BSA. Phage particles were added in a concentration of 1×10^{10} CFU/ml in PBS/0.1% Tween 20/0.1% BSA. Bound phage particles were detected with a peroxidase-conjugated rabbit anti-phage Ab (Amersham Pharmacia Biotech, Little Chalfont, U.K.). The reaction was developed with ABTS (Sigma-Aldrich, St. Louis, MO) as substrate. OD_{405–490} was measured in an ELISA reader (Dynatech, Denkendorf, Germany).

Mimicry ELISAs

ELISA plates (Nunc) were coated with trastuzumab (10 μ g/ml) in PBS, and blocked as previously described. Phage particles (10^8 CFU/well) and cell extracts (100 μ g protein/well) were added simultaneously and incubated for 1 h at 37°C and for 1 h at 4°C . After washing, bound phage was

detected as described for specificity ELISA. Percentage of specific inhibition was calculated relative to unspecific (sterical) inhibition incurred by the control cell extract.

A second setup consisted of Costar cell culture cluster plates (Corning, Corning, NY) coated with a suboptimal concentration of SK-BR-3 cells (1.5×10^5 cells/ml). Trastuzumab aliquots (0.1 μ g/ml) were preincubated with specific phage clones or wild-type phage at 1×10^9 – 10^{10} CFU/ml, then added onto the ELISA plates. Rituximab (0.1 μ g/ml) was used as negative control. Bound Ab was detected with a peroxidase-conjugated goat anti-human IgG Ab (Jackson ImmunoResearch Laboratories, West Grove, PA). The reaction was developed as previously discussed. Percentage of inhibition was calculated relative to the wild-type phage control.

All ELISA experiments were conducted at least three times. To confirm statistical significance of findings, χ^2 tests were performed.

Synthesis of vaccine constructs

As a paradigm, the peptide C-QMWAPQWGPDC was manufactured synthetically (piChem, Graz, Austria). Conformational accuracy was verified with trastuzumab in a DotBlot assay. In short, the peptide was solubilized in PBS/20% dimethylformamide, and dotted onto nitrocellulose membrane at 1 mg/ml. Blot strips were incubated with trastuzumab or rituximab, respectively, and bound Ab detected with a peroxidase-conjugated goat anti-human IgG Ab (Jackson ImmunoResearch Laboratories), using the ECL detection protocol (Amersham Pharmacia Biotech). Subsequently, the peptide was coupled via its C terminus to the immunogenic carrier, tetanus toxoid (TT), by the m-maleinimidobenzoyl-N-hydrosuccinimide ester method, and the conjugate again checked for trastuzumab binding capability in a DotBlot assay (as previously described).

Immunization of BALB/c mice

Two groups of BALB/c mice ($n = 7$) from the Institute for Laboratory Animal Science and Genetics (University of Vienna, Vienna, Austria) were immunized i.p. with 10 μ g of the mimotope conjugate (group A), or the carrier protein alone (group B), respectively, on days 1, 15, and 29. Aluminum hydroxide was used as an adjuvant in all groups. Blood was taken from the tail vein on days 0 (preimmune serum), 22, and 36. Mice were treated according to European Union Rules of Animal Care, with permission 66009/147/PR-4-2001 from the Austrian Ministry of Science.

Immunoblotting

Total cell lysates were separated by SDS-PAGE and immunoblots incubated with trastuzumab or serum pools as detecting Abs. Bound trastuzumab was detected by ^{125}I -labeled anti-human IgG (IBL), bound mouse Abs by ^{125}I -labeled anti-mouse Ig (Amersham Pharmacia Biotech).

Titers of sera were determined by incubation of serial dilutions with dotted TT, mimotope peptide, or blotted Her-2/neu, and bound Abs detected as previously described.

Fluorescence microscopy

SK-BR-3 cells were plated at low density on four-well Lab-Tek tissue culture chamber slides (Miles Laboratories, Naperville, IL), and grown overnight until half-confluent. They were then cooled to 4°C , washed with ice-cold PBS, fixed with 4% paraformaldehyde in PBS for 30 min, quenched with 50 mM NH_4Cl in PBS, and blocked with 1% BSA/PBS. Membranes were stained with rabbit anti-placenta alkaline phosphatase Ab, followed by Alexa 568-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR). Her-2/neu was detected with mAb 4D5 (kindly provided by Genentech, South San Francisco, CA), or with purified IgG (prepared with PROSEP-A spin columns; Millipore, Bedford, MA) from second immune sera (pooled from all mice in group A), followed by FITC-conjugated goat anti-mouse IgG (Caltag Laboratories, Burlingame, CA). Purified IgG of group B and preimmune sera were used as controls. Nuclei were stained with 0.1 μ g/ml Hoechst dye (Sigma-Aldrich) in PBS for 10 min. Cells were mounted in Mowiol mounting medium and viewed with a Zeiss Axioplan 2 (Carl Zeiss, Jena, Germany).

Transfection and internalization assay

SK-BR-3 cells were plated into chamber slides as previously described, and transfected with a Her-1/GFP construct (33), kindly provided by Dr. P. Bastiaens (EMBL, Heidelberg, Germany) using SuperFect Transfection Reagent (Qiagen), according to the manufacturer's instructions. After a 2-day expression period, cells were incubated for 15 min with 10 μ g/ml purified IgG from second immune sera of the mimotope-immunized and the control group, with trastuzumab, or with medium alone. Cells were fixed, mounted, and viewed as previously described.

Table I. Analysis of phage clones found positive in colony screening assays by DNA sequencing

No. of Biopanning Round	Positive Colonies/ No. Tested	Amino Acid Sequences Deduced from Positive Clones	Frequency ^a
1	0/120	—	—
2	13/175	C-QMWAPQWGPD-C C-KLYWADGELT-C C-VDYHYEGTIT-C	9 1 3
3	81/452	C-QMWAPQWGPD-C C-KLYWADGELT-C C-KLYWADGEFT-C C-VDYHYEGTIT-C C-VDYHYEGAIT-C	51 3 18 1 8

^a Number of times a particular sequence was found among the positive clones in a colony screening round.

Results

Biopanning and colony screenings

A constrained 10 mer random peptide phage display library was screened with trastuzumab. As a first indicator of successful biopanning, an increase of phage titers during rounds of panning was observed. The phage titer increased from 8×10^5 CFU/ml (first round) to 6.3×10^6 CFU/ml (second round) and finally to 4.3×10^7 CFU/ml (third round).

Colony screenings were performed after each round of panning. Phage clones reactive with trastuzumab, but not with the isotype-matched control Ab rituximab, were amplified and DNA sequenced. Five insert sequences were deduced from 94 positive phage clones (Table I). The insert C-QMWAPQWGPD-C was found most frequently, indicating high reactivity of the corresponding peptide with the trastuzumab paratope. The second most frequent sequence was C-KLYWADGEFT-C, followed by C-VDYHYEGTIT-C, C-VDYHYEGAIT-C, and C-KLYWADGELT-C.

Sequence analysis revealed no homology to Her-2/neu or any other member of the EGF receptor family in database alignments (EMBL Data Library). As the epitope recognized by trastuzumab is described to be discontinuous (34), this finding was expected.

Specificity ELISA

An ELISA was performed to confirm the colony screening results in a separate test system. All five mimotope candidates were specifically recognized by the selecting Ab trastuzumab, but not by the isotype-matched control Ab rituximab. Moreover, trastuzumab neither reacted with a circular 10 mer control peptide nor with wild-type phage (Fig. 1). Again, the peptide C-QMWAPQWGPD-C exhibited the highest reactivity with the Ab, corresponding to the colony screening result analysis. The other peptides did not parallel the colony screening results as much, but showed comparable binding intensities.

Mimicry tests

In a first setup, we tested three selected phage clones for mimicry with the Her-2/neu Ag. Phage particles and a cell extract containing Her-2/neu from the human breast cancer cell line SK-BR-3, or a Her-2/neu-negative control extract from MDA-MB-468 cells, were competing for ELISA plate-fixed trastuzumab. Results were reproducible with both cell lysate preparations. Only the SK-BR-3 cell extract was able to specifically displace bound phage from the Ab (Fig. 2). The three selected mimics, C-QMWAPQWGPD-C,

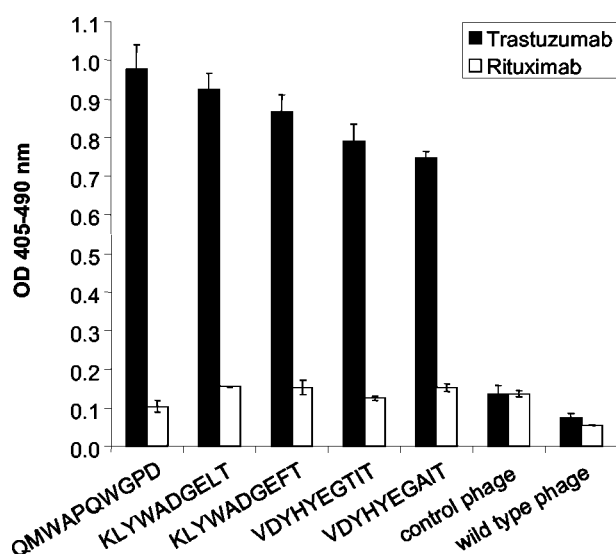


FIGURE 1. Trastuzumab specifically recognizes colony screening-positive phage clones in ELISA. Trastuzumab recognition of phage clones (■) displaying circular peptide inserts with sequences as given on the x-axis, of a phage clone displaying an irrelevant control peptide (control phage) or of wild-type phage, respectively. Isotype-matched control Ab (□) rituximab does not interact with trastuzumab mimotopes.

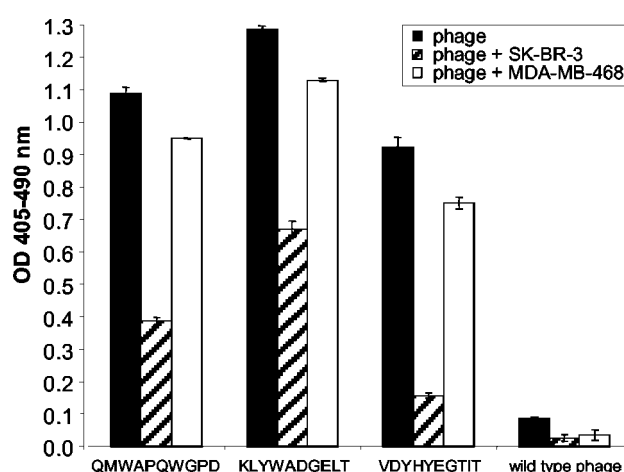


FIGURE 2. ELISA demonstrating mimicry of phage-displayed trastuzumab-specific peptides with Her-2/neu. Trastuzumab (■) recognition of phage clones displaying constrained peptide inserts with sequences as given on the x-axis or of wild-type phage, respectively, without competing substances. Her-2/neu (▨) in SK-BR-3 breast cancer cell extract competes with phage-displayed peptides for binding to coated trastuzumab, thereby reducing the number of bound phage particles. An extract (□) from the Her-2/neu-negative cell line MDA-MB-468 does not specifically interfere with trastuzumab-mimotope interactions.

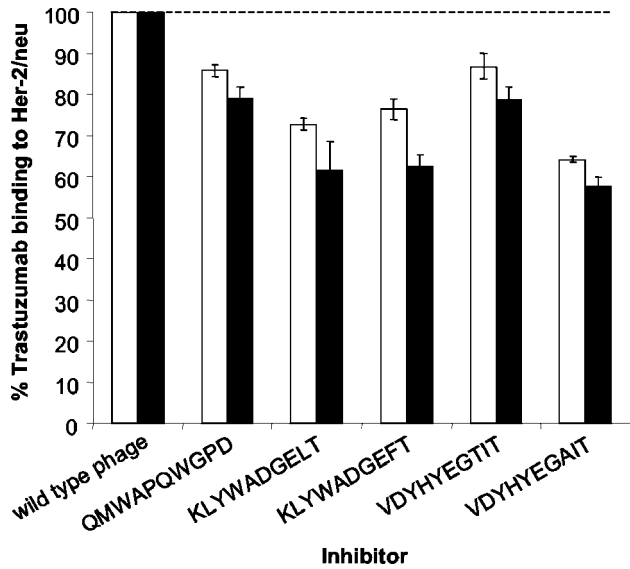


FIGURE 3. Trastuzumab-specific peptides displayed on phage inhibit Ab binding to its target Ag. The inhibition is phage-concentration dependent. Inhibition of trastuzumab binding (□) to Her-2/neu on coated SK-BR-3 cells by phage clones as indicated on the x-axis; phage concentration: 1×10^9 CFU/ml. Inhibition of trastuzumab binding (■) to Her-2/neu on coated SK-BR-3 cells by phage clones as indicated on the x-axis; phage concentration: 1×10^{10} CFU/ml.

C-KLYWADGEFT-C, and C-VDYHYEGTIT-C, were competitively removed by 59.4, 40.6, and 79.4%, respectively. Percentages were calculated relative to the nonspecific, possibly sterical, inhibition incurred by the control cell extract.

In an alternative setup, the ability of candidate phage clones to inhibit the binding of trastuzumab to Her-2/neu was examined. Impressively, all peptides interfered with this interaction. The inhibition was phage concentration-dependent, indicating the specificity of the observed phenomena. When using a phage concentration of 1×10^9 CFU/ml, inhibitions of 14.3% (C-QMWAPQWGPD-C), 27.3% (C-KLYWADGELT-C), 23.6% (C-KLYWADGEFT-C), 13.5% (C-VDYHYEGTIT-C), and 35.8% (C-VDYHYEGAIT-C) were attained. When using 1×10^{10} CFU/ml, inhibitions reached 20.0, 38.4, 37.3, 21.4, and 42.3%, respectively (Fig. 3). Complete inhibition could never be observed, due to the fact that a single mimotope represents only a part of the whole epitope and, moreover, that pIII display only offers three mimotope copies per phage particle for inhibition. All observed inhibitions were found to be statistically significant ($p < 0.01$).

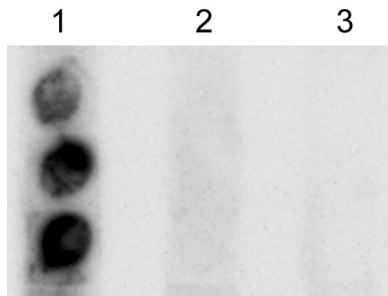


FIGURE 4. DotBlot showing specific recognition of the synthetic mimotope C-QMWAPQWGPD-C, dotted in triplicates, by trastuzumab (lane 1), but not by isotype control Ab rituximab (lane 2), or detecting Ab (buffer control, lane 3).

Table II. Ab titers elicited in BALB/c mice

	Group A (mimotope-TT)	Group B (TT)
Anti-TT	> 1/1,000,000	> 1/1,000,000
Anti-mimotope	1/100,000	0
Anti-Her-2/neu	1/10,000	0

Vaccine construct and immune response induced by the C-QMWAPQWGPD-C mimotope

Based on 1) the highest frequency of detection in colony screenings, 2) the highest intensity of recognition by trastuzumab in the specificity ELISA, and 3) the satisfying mimicry test results, the peptide C-QMWAPQWGPD-C was considered to be the most promising candidate for immunogenicity evaluation, and therefore manufactured synthetically. As a fourth criterium to proceed to immunizations, 4) a synthetic peptide must be specifically recognized by the selecting Ab. Indeed, synthetic C-QMWAPQWGPD-C was recognized by trastuzumab, but not by the isotype control (Fig. 4). Also conjugated to the immunogenic carrier, TT, mimotope conformation was preserved (data not shown). The immunogenicity of the mimotope conjugate was evaluated in BALB/c mice. All mice developed high anti-TT titers, indicating successful immunization in both groups. All seven mice in group A, immunized with the C-QMWAPQWGPD-C conjugate, showed a humoral response toward this peptide, and more importantly, also developed Abs recognizing Her-2/neu in a blotted SK-BR-3 cell lysate (Table II and Fig. 5).

Immunofluorescence

To demonstrate that the induced Abs also recognize Her-2/neu on the cell surface, we performed immunofluorescence staining. Monoclonal Ab 4D5, the murine archetype of herceptin, was used as a positive control (Fig. 6A). Purified IgG from sera of mice of group A showed intense staining of SK-BR-3 breast cancer cells (Fig. 6B), whereas purified IgG from mice of control group B, and preimmune sera, only caused background staining (Fig. 6, C and D, respectively). For comparability, membranes and nuclei were stained in the same way.

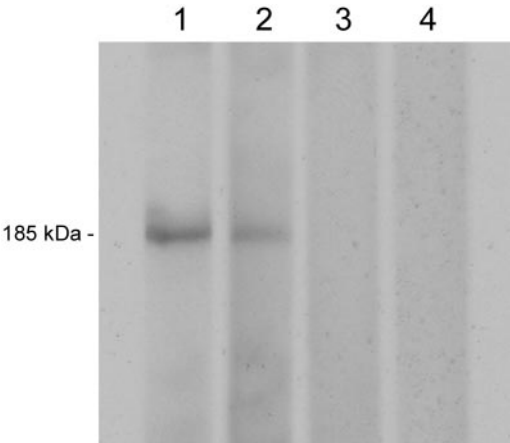


FIGURE 5. Immunodetection of Her-2/neu in a Western blot of a SK-BR-3 cell lysate. Blotted Her-2/neu is detected by trastuzumab as a band at 185 kDa (positive control, lane 1). Sera from mice immunized with a Her-2/neu-mimotope-TT conjugate also recognize Her-2/neu (lane 2), whereas sera from mice immunized with the carrier TT alone (lane 3) show no reactivity with the Ag. Buffer control is shown in lane 4.

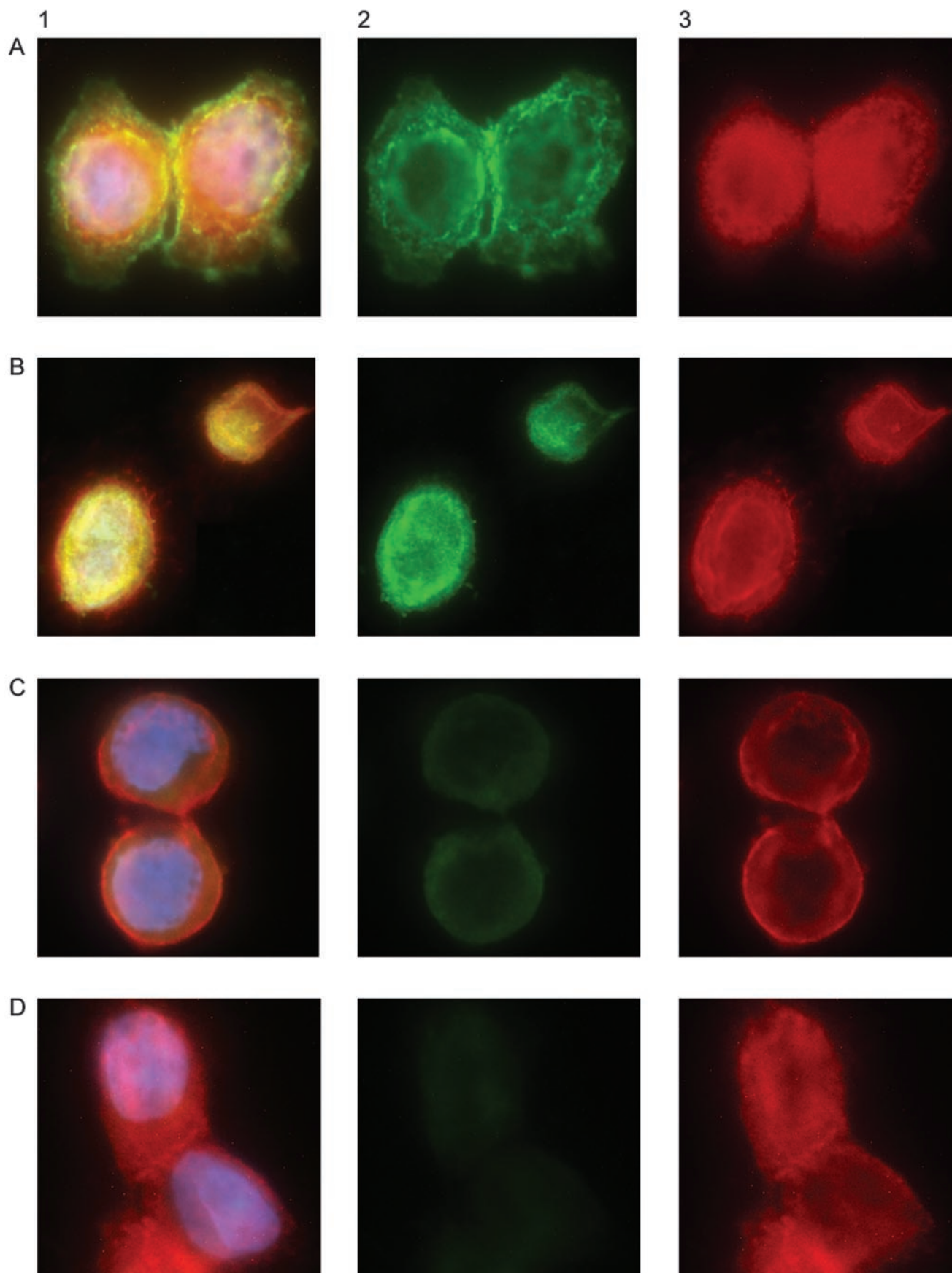


FIGURE 6. Specific recognition of Her-2/neu by anti-mimotope sera in immunofluorescence staining of SK-BR-3 breast cancer cells. *A*, 4D5 positive control is shown in (panels 1–3). *B*, Purified IgG from mimotope-immunized mice specifically recognizes cell surface Her-2/neu Ag (panels 1–3). *C*, Purified IgG from mice immunized with the carrier protein alone (panels 1–3) and preimmune serum (*D*, panels 1–3) only show background staining. *A–D*, Ab staining (green), membrane staining (red), and nuclear staining (blue) are shown (column 1). FITC channel (column 2) and Texas Red channel (column 3) also shown.

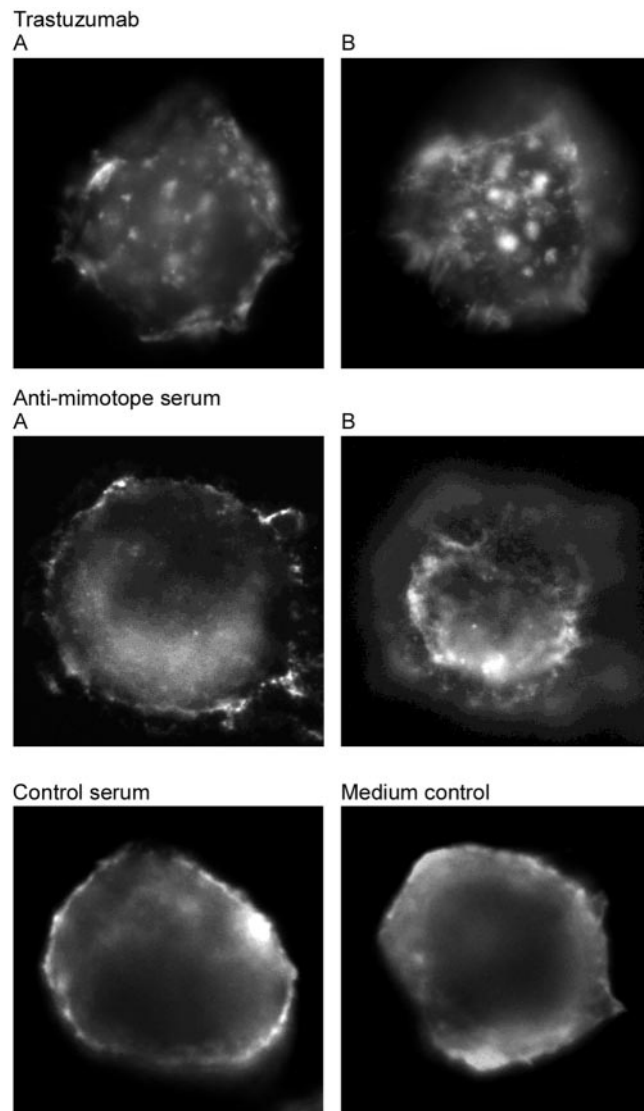


FIGURE 7. Internalization and perinuclear vesicular distribution of Her-2/neu-Her-1 heterodimers after incubation with anti-mimotope sera. Trastuzumab positive control (*top panels*) at membrane level of focus (A) and at vesicle level of focus (B); (*middle panels*) show purified IgG from mimotope-immunized mice causes internalization and a perinuclear vesicular staining, indicating receptor complexes to be localized in late endosomes/lysosomes; membrane level of focus (A) and vesicle level of focus (B); (*bottom panels*) show purified IgG from mice immunized with the carrier protein alone (control serum) does not lead to a change in the surface localization of the receptor, as seen in untreated cells (medium control).

Internalization assay

The antitumor activity of trastuzumab and other tumor-inhibitory anti-Her2/neu Abs was shown to be the result of internalization of the receptor-Ab complex and movement into endocytic vesicles (3, 15, 19). Consequently, we performed an internalization assay to assess whether the Abs induced by the mimotope have a similar biological activity. To visualize the internalization of the receptor, we transfected SK-BR-3 cells with GFP-labeled Her-1. Her-1 was chosen because it is known to form heterodimers with Her-2/neu, which are internalized together (3). SK-BR-3 cells have very low constitutive levels of the Her-1 receptor, so no unlabeled receptors could interfere in the assay, as would have been the case with a GFP-labeled Her-2/neu. Furthermore, transfection of SK-BR-3

cells with Her-1 also ensures a high rate of Her-1-Her-2/neu heterodimer formation.

After a 15 min incubation with either trastuzumab or purified IgG of mice from group A, a reduction of surface staining and the appearance of vesicles was observed (Fig. 7, *top panels*). The perinuclear distribution of the vesicular staining suggests late endosomes/lysosomes and thus entering of a degradation pathway. The control IgG, purified from mice immunized with the carrier alone, showed no internalization, but only surface staining, as did the cells treated with no Ab at all (Fig. 7, *bottom panel*).

Discussion

To date, various immunological strategies targeting the Her-2/neu oncoprotein have been used, including vaccination with its ectodomain (35), immunization with peptides derived from the extracellular portion of the molecule (36–38), as well as vaccination with DNA (39). These concepts all primarily aimed at the induction of cellular anti-Her-2/neu responses, with some of them yielding impressive results (36–38). However, the clinical efficacy of anti-Her-2/neu Abs and the observation that some patients demonstrate an endogenous B cell response to Her-2/neu (10, 40) suggest that not only the induction of cellular immunity, but also of a strong and targeted humoral immune response is possible and might be biologically important. The successes seen with trastuzumab treatment strengthen this point of view. An active immunization resulting in the induction of trastuzumab-like Abs could elicit long-lasting humoral immune responses, which potentially might be more effective than passive applications of the Ab. Another major advantage of vaccination lies in the establishment of immunological memory against the Ag. Moreover, major obstacles of passive administration of Igs, such as a comparatively short half-life, unequal tissue distribution, and the inherent immunogenicity of high doses and prolonged administration of even humanized mAbs, would be overcome by active immunization.

As the tertiary structure of Her-2/neu was described only recently (41), B cell epitopes for the design of inhibitory vaccines could only be predicted by applying complicated algorithms. But even so, these peptide regions are only estimates of possible conformational epitopes, and may still induce growth-enhancing Abs (42). Moreover, the Her family receptors are known to be glycosylated, and glycosylation has been shown to play a decisive role in the immunogenicity of tumor-associated Ags. This possibly explains the difficulties met by computer-aided epitope searches.

In this situation, the phage display technique is an interesting tool. It is a relatively new technique to define peptides mimicking natural epitopes, including conformational B cell epitopes (43), as is the case with the epitope recognized by trastuzumab (34). The Ag to be mimicked can even be a carbohydrate structure (44). Phage display peptide libraries consist of filamentous phage particles displaying random peptides of defined length on their surface, which are either fused to the phage minor coat protein pIII, or at a higher copy number, to the major coat protein pVIII. By biopanning (31) of phage display libraries, mimotopes can be selected. These are peptides from 6 to 28 amino acids in length, assembled either in linear or in constrained (circular) form.

In this study, we generated mimotopes of a known inhibitory epitope on Her-2/neu, the epitope recognized by trastuzumab. For this purpose, we chose a constrained 10mer phage library because of the favorable stability characteristics of these short circular inserts. Trastuzumab was used to select matching peptides from a repertoire of $>10^9$ possible ligands. The surface characteristics of these mimetic peptides are equivalent to the epitope of the selecting Ab, although their amino acid sequence may differ (45), as seen with the five mimotopes presented in this study. This finding

is expected when selecting mimotopes for a conformational epitope, as only a small number of amino acids mimic a region composed of several polypeptide chains in the original Ag.

Mimicry tests are mandatory to designate a peptide as a mimotope. After the reactivity of the candidate peptides to the selecting Ab is confirmed in colony screenings and specificity tests, the structural equivalence to the original Ag has to be ascertained. All five candidate peptides in this study showed significant mimicry with Her-2/neu in two experimental setups. Only a cell extract containing Her-2/neu was able to displace phage displayed mimotopes from bound trastuzumab. In the second setup, selected peptides inhibited the Ab binding to Her-2/neu. Thus, we demonstrate that the described mimotopes represent true mimics of the trastuzumab epitope.

Mimotopes are suitable for the induction of an epitope-specific humoral immune response as, for instance, previously reported in the field of type I allergies (46) and also for possible vaccinations against malignant melanoma (47, 48). Both are areas in which epitope-specific induction of Abs is crucial, as otherwise symptoms can be aggravated or malignant growth enhanced. As sequences are easily obtained, mimotope peptides can be synthesized custom-made. Synthetic peptide vaccines are cost-effective, simple to produce and can easily be quality-controlled during the manufacturing process. They are chemically stable and contain no oncogenic, toxic, or infectious material. As active immunogens, synthetic mimotopes can induce continuously available tumor-targeting Abs. We demonstrate in this study that our mimotope vaccine is immunogenic and elicits Igs recognizing the original Ag, Her-2/neu. This was shown in Western blot and also in immunofluorescence experiments. In the latter assay, the induced anti-mimotope Abs caused cell membrane staining of the same pattern and intensity as the positive control Ab 4D5, the murine precursor of trastuzumab. As tumor growth inhibitory anti-Her-2/neu Abs are described to cause receptor internalization and accelerated degradation (3, 15, 19), we performed an internalization study. Indeed, the induced Abs showed the same vesicular staining pattern as trastuzumab, indicating similar biological activity.

Undoubtedly, the effects of induced Abs by mimotope vaccination will have to be closely monitored, even though the danger of tumor growth stimulating Abs was ruled out by deliberate epitope selection with the phage display technique. Although no toxicities could be observed in mimotope-immunized animals so far, the potential occurrence of cardiotoxicity, which is known to occur as a complication of trastuzumab administration (49), must be assessed. Nevertheless, the benefits of a narrowly focused active immune response against a major cancer Ag as Her-2/neu are expected to outweigh possible disadvantages by far.

In conclusion, we demonstrate trastuzumab mimotopes to be novel vaccine candidates. They are immunogenic and elicit Abs recognizing the original Ag, Her-2/neu. Moreover, these Abs also showed a trastuzumab-like activity in receptor internalization induction. Their major advantage is to focus a long lasting humoral immune response to a therapeutically relevant epitope. Sustained availability of growth-inhibitory anti-Her-2/neu Abs may complement previous trials, which aimed at other tumor characteristics. The active induction of Abs with qualities like trastuzumab may lead to the mimotopes' use in disease prevention, adjuvant treatment, and therapy of advanced disease.

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