

Endogenous Anti-HER2 Antibodies Block HER2 Phosphorylation and Signaling through Extracellular Signal-Regulated Kinase

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Abstract

Immunologic targeting of the oncoprotein HER2/*neu* with monoclonal antibodies is an important component of current therapeutic strategies for patients with locally and systemically advanced breast cancer. Engineered antibodies targeting HER2 may have agonist or antagonist effects on HER2, but little is known about whether endogenous antibodies modulate HER2 activity. Vaccination of patients with HER2 peptides successfully induced antibodies in a minority of patients with HER2-expressing malignancy. A subset of antibodies specifically suppressed phosphorylation of HER2 on tyrosine Y1248, a residue critical for HER2 signaling through extracellular signal-regulated kinase. These antibodies also suppressed extracellular signal-regulated kinase phosphorylation and inhibited colony formation in soft agar. The majority of the antibodies that suppressed HER2 phosphorylation displayed specificity for amino acids 328 to 345 and 369 to 384. The isotype of anti-HER2 antibodies was predominantly IgG3 of low avidity, suggesting a Th1 response to peptide vaccine. Endogenous anti-HER2 antibodies can effectively suppress HER2 kinase activity and downstream signaling to inhibit the transformed phenotype of HER2-expressing tumor cells. (Cancer Res 2005; 65(2): 650-6)

Introduction

The oncoprotein HER2/*neu* is overexpressed in many epithelial malignancies and is an established target for antibody and vaccine therapies. HER2 is constitutively activated in the absence of ligand and signaling occurs through phosphorylation of specific tyrosine residues which serve as docking sites for the adaptor molecules SHC and GRB2 with subsequent activation of Ras-ERK and PI-3K pathways (1–3). HER2 activation of the extracellular signal-regulated kinase (ERK) pathway is primarily dependent on phosphorylation of Y1248/1253 in the intracellular domain of HER2 and mutation of this residue substantially diminishes the transforming potential of HER2 (1). Targeting of HER2 with monoclonal antibodies is one means of suppressing HER2-induced transformation and these antibodies mediate an array of effects on tumor growth and signal transduction. These include antibody-dependent cell-mediated cytotoxicity, down-regulation of cell surface HER2, inhibition of receptor heterodimerization of HER2 with other epidermal growth factor receptor family members, and suppression of cell cycle progression by induction of p27Kip1 (4–8). A number of groups have shown that patients with

HER2-expressing malignancies develop endogenous antibodies to HER2 (9–11). Little is known about the effects of these endogenous antibodies on phosphorylation of HER2 and its downstream signaling. We explored the effects of antibodies induced by HER2 peptide vaccines on HER2 phosphorylation, signaling through the ERK/mitogen-activated protein kinase pathway and proliferation of HER2-expressing breast cancer cell lines.

Materials and Methods

Patient Samples. Patients with HER2-positive stage III or IV breast or ovarian cancer were eligible for a phase I Institutional Review Board-approved study of HER2 peptide-based vaccine. Forty-five patients received one of three vaccines: (1) ECD vaccine containing peptides p42-56, p98-114, and p328-345; (2) ICD vaccine containing peptides p776-790, p927-941, and p1166-1180; or (3) HLA-A2 vaccine, containing peptides p369-384, p688-703, and p971-984. Vaccines were given as previously described (12).

ELISA. Capture ELISA for protein-specific responses was done as previously reported using an IgG-specific secondary antibody (11). Peptide-specific responses were assessed using 96-well Immulon 4 plates (Dynatech Laboratories, Chantilly VA) incubated overnight at 4°C with HER2 peptides at a concentration of 10 µg per well. Wells were blocked with PBS and 1% bovine serum albumin, 100 µL per well for 4 hours at room temperature. The wells were incubated with patient sera diluted 1:100 and 1:200 for 1 hour at room temperature. Goat anti-human IgG was added to the wells at a 1:5,000 dilution in PBS/1% bovine serum albumin and incubated for 45 minutes at room temperature. TMB developing reagent is added and the reaction stopped with 1 N HCl and the absorbance read at 450 nm. Antibody levels were quantitated by comparison to a standard curve. Positive HER2 protein or peptide-specific antibody levels were defined as previously described (13).

Flow Cytometry. HER2-expressing, SK-BR-3 breast cancer cells, and a HER2-negative MCF-7 subline were incubated with patient sera at 1:20 dilution at saturating concentrations followed by FITC-conjugated goat antihuman F(ab')₂ serum (Pierce, Rockford, IL). Each incubation was done for 60 minutes at 4°C. After the final wash, cells were fixed with 2% formaldehyde-PBS. Cells were analyzed by flow cytometry with a FACSCalibur instrument system (Becton Dickinson, San Jose, CA).

Antibody Isolation. Serum from patients with HER2-specific antibodies as defined by ELISA was separated by fast protein liquid chromatography over Protein-G Sepharose (Amersham, Sunnyvale, CA). Eluted immunoglobulin fractions were brought to pH 7.4, checked for purity by silver stain, concentrated, and assayed for protein concentration.

Western Analysis. Antibodies were to phosphotyrosine (4G10, Upstate Biotechnology, Lake Placid, NY) phosphoHER2 (Cell Signaling, Mountain View, CA), HER2 7.16.4 (Ab-4, Oncogene Sciences, Cambridge, MA), HER2 TA-1 (Ab-5 Oncogene Sciences), ERK and phosphoERK (Cell Signaling), and to tyrosine phosphorylated residues of HER2 (Ab 2247, 2249, 2241, Cell Signaling). The phospho-HER2 site-specific antibodies utilized in this study were generated against peptides phosphorylated on the residues corresponding to Y1248, Y1221, and Y877. Antibodies undergo negative and positive selection, bind specifically to phosphorylated HER2, and do not bind to unphosphorylated peptide. Thirty micrograms of protein were loaded into an 8% to 16% gel, run, and transferred to nitrocellulose. The membranes were blocked overnight at 4°C in 5% milk/PBS. Antibodies were added at a 1:1,000 dilution for 1 hour in 3% bovine serum albumin/PBS. Anti-mouse horseradish

Note: Patient care was conducted through the Clinical Research Center Facility at the University of Washington (MO1-RR-00037).

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peroxidase antibody (Pierce) was added at 1:2,000 dilution for 30 minutes. Signal was detected with enhanced chemiluminescence Plus (Amersham).

Colony Assays. SK-BR-3 were exposed to indicated antibodies for 4 hours at 10 $\mu\text{g}/\text{mL}$, trypsinized, and plated at 10,000 cells per well in 6-well plates in 1% agarose. Colonies larger than 60 μm were counted at 14 days. Data points represent average \pm SD for triplicate wells. All assays were done thrice.

Evaluation of Anti-HER2 Antibody Avidity and Isotype. HER2-specific antibody levels were quantitated and avidity and isotype specificity was evaluated as previously described (14, 15). Two-fold serial dilutions of serum samples were plated in duplicate and incubated for 3 hours at room temperature. After four washes, 200 μL of wash buffer containing 8 mol/L urea was added to one of the duplicate wells and incubated at room temperature for 3 minutes. All wells were washed four times. For the detection of antibody binding the plates, goat anti-human IgG1, IgG2a, IgG3, and IgG4 conjugated with horseradish peroxidase (diluted 1:10,000; Zymed Laboratories, South San Francisco, CA) was added for 45 minutes at room temperature. After washing, 3,3',5,5'-tetramethylbenzidine substrate solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added, and the reaction was allowed to proceed for 3 to 5 minutes and was stopped by adding 75 μL of 1 N HCl per well. For a given patient serum dilution, the avidity index was calculated using the following formula: (change of OD with urea/change of OD without urea) \times 100; and the avidity index was expressed as a percentage. An avidity index <30% is considered low avidity, 31% to 50% is considered intermediate avidity, and >51% is considered high avidity. Avidity is reported here at the 1:25 dilution of sera.

Results

Endogenous HER2-Specific Antibodies Bind Cell Surface HER2. Eleven patients entered on HER2 peptide vaccine studies developed antibodies with specificity to the HER2 protein during the course of vaccination as defined in Materials and Methods. To determine if peptide-induced antibodies could effectively recognize native HER2, we assayed the binding of antibodies to cell surface HER2. The HER2-positive breast cancer cell line SK-BR-3 was utilized as the positive control target and a clone of MCF-7 with low HER2 expression was used as the negative control target. Serum from patients and controls without detectable anti-HER2 antibodies was used as negative control samples. Analysis of representative samples is shown in Fig. 1. Antibodies from all patients were able to bind HER2 in its native conformation on the cell surface (Fig. 1A) and did not bind to the HER2 negative control cells (Fig. 1B). Essentially, all positive patient sera bound with the intermediate fluorescence shown in Fig. 1A.

The Effect of HER2-Specific Antibodies on HER2 and ERK Phosphorylation. One mechanism by which antibodies might inhibit cell proliferation and transformation is by blocking activation of HER2 at the cell surface, thereby suppressing effective downstream signal transduction. Antibodies from serum samples collected prior to vaccination and at the completion of the vaccination course were purified by fast protein liquid chromatography and incubated with SK-BR-3, a HER2-expressing human breast cancer cell line for 1 hour and whole protein lysates analyzed for HER2 phosphorylation on residues 877, 1221, and 1248. Antibodies obtained from postvaccination serum from 6 of 11 patients suppressed Y1248 phosphorylation specifically (Fig. 2A). One patient's serum contained antibodies which suppressed HER2 phosphorylation prior to vaccination and antibodies isolated after vaccination were able to further suppress Y1248 phosphorylation (sample 4). Phosphorylation of phosphoresidues Y877 and Y1221 was unaffected by antibodies or by exposure to the control antibody *c-neu* 5 which up-regulates HER2 phosphorylation.

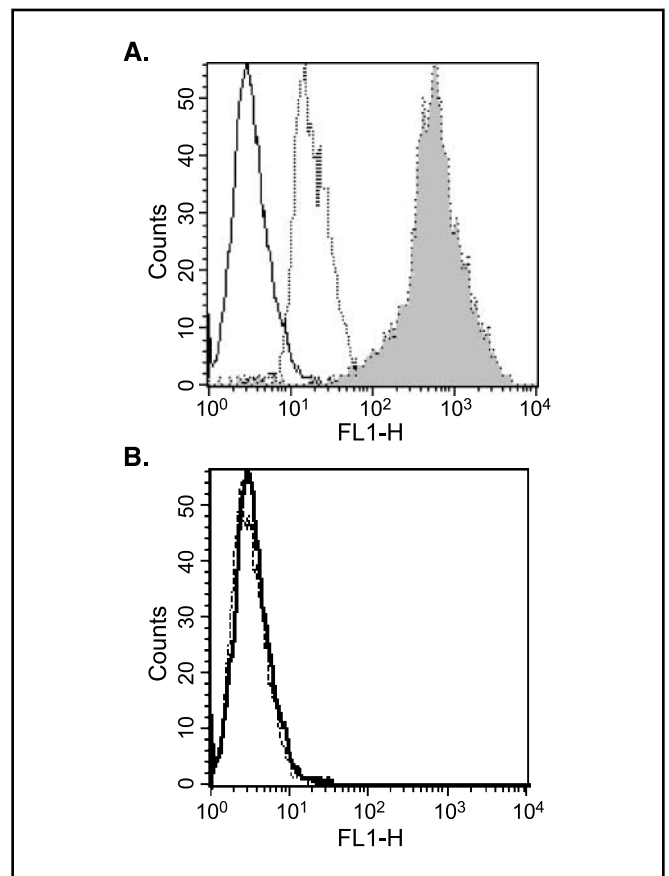


Figure 1. Endogenous anti-HER2 antibodies bind native, cell surface HER2. A, HER2-positive SK-BR-3 cells were incubated with *c-neu* 5 monoclonal antibody to HER2 (shaded), sera from a patient vaccinated with HER2 peptide (dashed line), or control sera (solid line) and antibodies detected with FITC-labeled secondary antibody. B, HER2-negative MCF-7 breast cancer cells were incubated with control sera without HER2 antibodies (solid line) and patient sera containing anti-HER2 antibodies (dashed line).

The Y1248 phosphoresidue regulates association of SHC with HER2 and mutation or deletion of Y1248 abrogates downstream ERK activation (1). For this reason, we then assessed the effects of these antibodies on ERK activation in the same lysates and showed that four of these patients had antibodies induced by vaccination which suppressed ERK activation without suppressing total cellular ERK levels (Fig. 2A).

Time Course of HER2 Antibody Effects on HER2 and ERK Phosphorylation. To better delineate the effect of these antibodies on HER2 activation, the effects of suppressive antibodies were further characterized. Incubation with antibodies from patients 1 and 2 suppressed HER2 phosphorylation maximally by 60 to 120 minutes with a return to near baseline after 6 hours (Fig. 2B). These antibodies also suppressed ERK phosphorylation by 60 to 120 minutes and suppression was maintained for at least 24 hours. Because HER2 transformation may also be mediated by activation of the PI-3K pathway, we probed the same lysates with antibodies to phosphorylated AKT, but there were no changes in phosphoAKT levels over the same time interval (data not shown).

Effect of Induced Antibodies on Colony Formation. The effect of antibodies on anchorage-independent growth in semisolid agar, a standard assay for the transformed phenotype, was tested from a select number of samples for which adequate amounts of paired antibodies were available. Three of the

antibodies, which suppressed both HER2 phosphorylation and ERK activation (samples 1, 2, and 7), were tested in parallel with antibodies from patients whose antibodies suppressed ERK phosphorylation without affecting HER2 (sample 3) or had no effect on phosphorylation of either HER2 or ERK (samples 6 and 10). The antibody 7.16.4 (*c-neu* 5), a known inhibitor of HER2

mediated transformation, suppressed colony formation of SK-BR-3 to 40% of vehicle treated control. The induced antibodies, which showed inhibition of both HER2 and ERK phosphorylation, effectively blocked colony formation to between 30% and 60% of control (Fig. 3), whereas antibodies from prevaccination sera showed no effect. Antibodies purified from a control patient

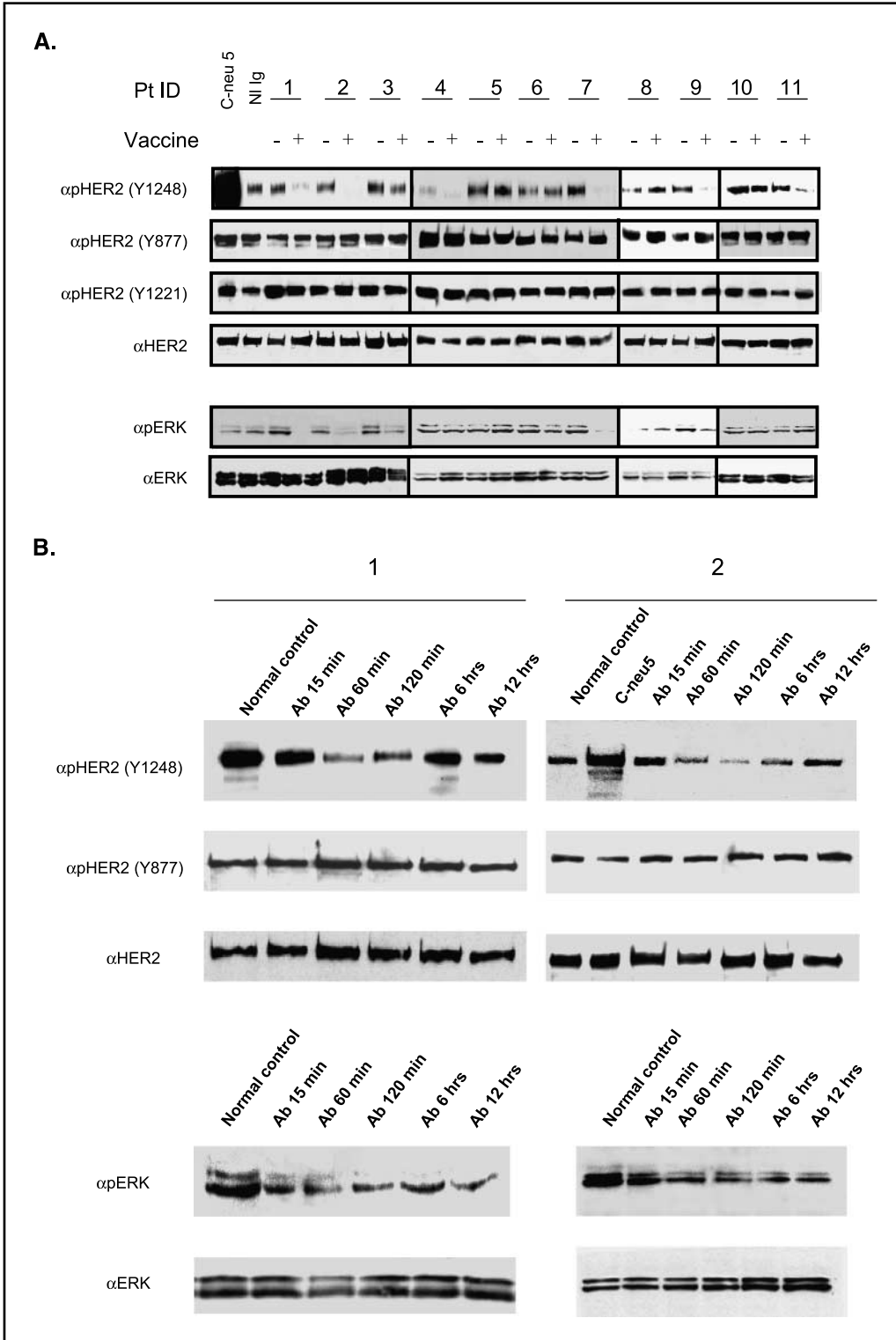


Figure 2. Effects of induced anti-HER2 antibodies on HER2 and ERK phosphorylation. Patient sera were procured before (-) and after (+) HER2 peptide vaccination. Antibodies were purified over by Protein A chromatography and used to treat HER2-expressing SK-BR-3 breast carcinoma cells in culture. Control and patient antibodies were used at 10 μ g/mL for 1 hour (A) or antibodies from patients 1 and 2 for indicated periods (B) and protein from lysates analysed by Western with antibodies specific for tyrosine phosphorylation on residues 877, 1221, and 1248 (*pHER2*) and for total HER2 levels. The same samples were run in tandem and probed with antibodies to phosphorylated ERK (*pERK*) or total ERK.

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also had no significant effect on the number of colonies. The clear difference in effect on growth in semisolid agar between prevaccination and postvaccination antibodies show that the ability to suppress HER2 phosphorylation is capable of modulating downstream signaling and phenotypic transformation, which is dependent on HER2 function.

Specificity of Induced Anti-HER2 Antibodies. The specificity of antibodies induced by HER2 peptide vaccination was tested against the panel of vaccine peptides to determine if suppression of phosphorylation and ERK signaling was associated with generation of immunity to a domain of HER2 used in the vaccine. All of the antibodies, which suppressed ERK phosphorylation, reacted with peptides containing amino acids 328 to 345 or 369 to 384 (Table 1). Patient sera often contained antibodies with specificity to other peptides spanning the extracellular domain (amino acids 1-652) or intracellular domain (amino acids 682-1255), although there was no association with inhibition of HER2 phosphorylation or ERK with antibodies to the other domains of HER2.

Isotype and Avidity of Induced Anti-HER2 Antibodies. This peptide based vaccine strategy using granulocyte macrophage colony-stimulating factor as adjuvant induces T-cell responses in >90% of patients completing vaccination (12). Although this strategy was not specifically designed to induce antibodies, characterizing the immunoglobulin isotypes induced by the vaccine and the level of induction of each isotype may help to both define Th1 versus Th2 help induced by this approach, as well as to direct subsequent approaches to generating HER2 antibodies. We evaluated the isotype class of IgG antibodies induced in these patients and characterized avidity for the bulk antibody as previously described (14, 15). Some samples could not be adequately analyzed due to insufficient amounts of serum (samples 7 and 10). As shown in Table 2, high levels of HER2-specific IgG can be achieved in a subset of patients, with HER2-specific IgG ranging from 1.2 up to 9 $\mu\text{g}/\text{mL}$. Most of the responses were of the IgG3 isotype and were of low avidity, suggesting induction of a Th1 response, and consistent with the effective induction of T-cell help. The samples with the highest levels of HER2-specific IgG had the greatest effect on HER2 phosphorylation (Table 2; samples 1, 2, 3, 7, and 9).

Discussion

The induction of target-specific antibodies is an important component of successful vaccine strategies. The generation of effective antitumor immunity to HER2-expressing malignancies requires HER2-specific antibodies and CTLs to completely block tumor formation in some models and significantly improves vaccination efficacy in others (16, 17). In some models, induction of antibodies alone is sufficient for protection from implantation with HER2-expressing tumors and rejection of established tumors (18). The studies reported here show that vaccination in patients induces antibodies which suppress HER2 phosphorylation and downstream activation of ERK. ERK is crucial to HER2-mediated transformation, and although a number of oncogenes can transform mammary epithelium in animal models, HER2 specifically activates the ERK/mitogen-activated protein kinase pathway, and relies on this pathway to mediate transformation (19). Inhibitors of mitogen-activated protein kinase kinase, the upstream activator of ERK, block phenotypic transformation by HER2 in the same model, confirming the importance in ERK signaling for transformation (19). Other pathways, which are implicated in HER2 mediated transformation, also depend on ERK activity, such as induction of cyclin D and p21Cip1 (20, 21). Although other signal transduction pathways are clearly important in HER2-mediated transformation, activation of ERK is a consistent downstream consequence of HER2 expression and is generally considered the principal driver of cell proliferation (22). The ability of these antibodies to suppress ERK activity has other implications as well, because suppression of ERK in HER2-expressing cells restores sensitivity to tamoxifen (23). This seems to be related to the ability of HER2 activation of ERK to suppress estrogen receptor function and ERK inhibition to restore estrogen receptor expression (24). Agonistic antibodies, which activate HER2 phosphorylation, stimulate ERK activity and increase measures of transformation *in vitro* (25, 26).

In this study, we found that the ability of vaccine-induced antibodies to inhibit HER2 phosphorylation correlated well with suppression of downstream ERK activity and subsequent suppression of proliferation. However, antibodies isolated from two samples suppressed HER2 phosphorylation with limited effect on ERK activation, and antibodies from one sample partially suppressed

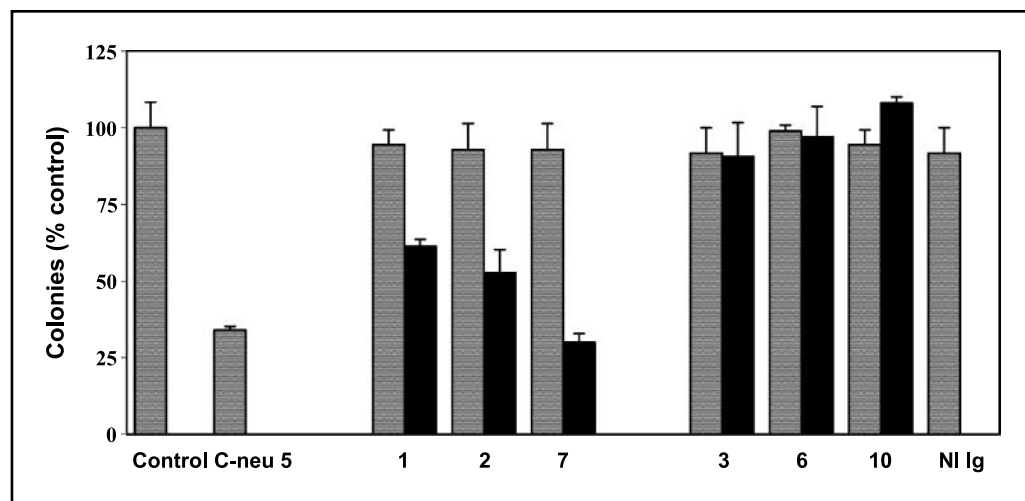


Figure 3. Effects of induced anti-HER2 antibodies on colony formation. HER2-expressing SK-BR-3 breast carcinoma cells were treated with antibodies from prevaccination and postvaccination serum for 4 hours at 10 $\mu\text{g}/\text{mL}$, trypsinized and plated at 10,000 cells per well in 6-well plates in 1% agarose containing antibody at 10 $\mu\text{g}/\text{mL}$. Colonies larger than 60 μm were counted at 14 days. Columns, average for triplicate wells; bars, \pm SD. All assays were done thrice.

Table 1. Summary of peptide specificity of anti-HER2 antibodies

Sample	HER2 amino acid peptide sequence									HER2 IgG levels
	42-56	98-114	328-345	369-384	688-703	776-790	927-941	971-984	1166-1180	
1	—	+	+	—	+	—	—	+	+	9.26
2	+	+	+	—	—	+	—	—	—	4.41
3	—	—	—	+	—	—	—	—	—	8.40
4	—	—	—	+	—	—	—	—	—	1.81
5	—	—	—	—	—	—	—	—	—	1.90
6	—	—	—	+	—	+	+	—	—	8.00
7	—	—	—	+	—	—	—	—	—	2.00
8	+	+	+	+	+	+	+	+	—	1.54
9	—	—	—	—	—	—	—	—	—	2.50
10	—	—	+	+	—	+	—	—	—	1.20
11	—	—	+	—	—	—	—	—	—	1.20

NOTE: Specificity for serum antibodies from patients with documented anti-HER2 responses to ELISA was determined, and a positive response was determined as defined in Materials and Methods.

ERK activity without having any effect on cell proliferation (Fig. 2A). Monoclonal antibodies may suppress HER2-mediated proliferation *in vitro* through inhibition of dimerization, down-regulation of cell surface HER2 (leading to reduced signaling), by inhibiting interactions with downstream signaling molecules such as SHC or GRB2 or by changing the conformation of homodimers or heterodimers within the cell membrane. If HER2 phosphorylation is suppressed without suppressing dimerization with HER3, generally considered the most active heterodimer with regard to proliferation (27), signaling may continue. Antibodies with different epitopes could potentially change the orientation of HER2 homodimers or heterodimers to one another, substantially changing phosphorylation and downstream signaling. As proposed by Yip et al. (25), antibodies to HER2 are predicted to change the rotational

conformation of dimers relative to one another and in this way either inhibit or activate HER2 signaling. If antibodies suppress intramolecular phosphorylation without down-regulating membrane localization, effective suppression of ERK signaling may not occur. Suppression of ERK may require that a threshold be reached to adequately suppress anchorage independent growth (sample 4), accounting for the lack of effect for this single sample. Despite these caveats, the ability of endogenous antibodies to suppress ERK signaling is an important and relevant mechanism for inhibition of cell proliferation.

Herceptin (trastuzumab) is a humanized monoclonal IgG1 antibody, which binds to amino acids 529 to 625 in the juxtamembrane region of HER2. In preclinical models, trough levels of 20 µg/mL are the minimum necessary for efficacy and levels in patients

Table 2. Summary of avidity and isotype of anti-HER2 antibodies

	Immunoglobulin levels (µg/mL)					% Avidity
	HER2 IgG	IgG1	IgG2	IgG3	IgG4	
1	9.26	0.00	0.00	1.84	0.38	1.3
2	4.41	1.94	0.00	1.83	0.00	17.9
3	8.40	4.66	1.96	0.85	0.38	9.6
4	1.81	0.00	0.00	0.25	0.00	1
5	1.90	0.00	0.00	1.34	0.00	2
6	8.00	3.14	3.56	0.93	0.00	3.4
7	2.00					6
8	1.54	0.00	0.00	0.64	0.26	2.3
9	2.50	0.00	2.49	0.93	0.15	2.1
10	1.20					—
11	1.2	0	0	0.88	0	1

NOTE: Levels of antibody, avidity, and isotype of serum antibodies from patients with anti-HER2 responses was determined as defined in Materials and Methods. Inadequate amounts of serum were available to fully characterize some samples.

receiving weekly therapy average 70 $\mu\text{g}/\text{mL}$ (28). *In vitro*, herceptin induces minimal effects on HER2 phosphorylation when analysis is carried out under denaturing conditions (29). Herceptin down-regulates membrane-associated HER2 with subsequent suppression of ERK, ultimately resulting in cell cycle arrest (30, 31). *In vivo* it seems that antibody dependent cellular cytotoxicity plays a significant role in herceptin's inhibitory effects on HER2 expressing cells, as in transgenic animals lacking Fc receptors efficacy is reduced by $\sim 60\%$ (4). The antibodies described in this study were principally of the IgG3 isotype and were able to achieve rapid suppression of HER2 phosphorylation and ERK activation within 15 minutes using concentrations of 10 $\mu\text{g}/\text{mL}$, which are comparable to serum levels of herceptin used in clinical practice. Antibodies induced by vaccination differ substantially in their effect on HER2 phosphorylation, suggesting significant differences in mechanism of effect.

The antibodies which suppressed HER2 phosphorylation specifically bind to peptides incorporating amino acids 328 to 345 or 369 to 384, portions of the extracellular domain predicted to induce T helper activity. Other investigators have shown that in animal models, peptides targeting amino acids 316 to 339 and 378 to 398 of HER2 effectively induced antibodies which blocked proliferation and metastasis of HER2-expressing cell lines (32, 33). These domains are in close proximity or overlap with the peptide specificity of the endogenous antibodies characterized in this study, suggesting that this region of the extracellular domain is a particularly important region of HER2 to consider in vaccines targeting humoral immunity to HER2. Monoclonal antibodies raised to different portions of HER2 may have inhibitory or activating effects and this has raised the specter that induction of antibodies *in vivo* might even be harmful by stimulating proliferation in target cells in which HER2 kinase activity is activated (26). We did not find any evidence of HER2 activation by these antibodies, nor have we found HER2 stimulatory activity in a limited number of HER2-specific antibodies isolated from patients with HER2-expressing malignancies (data not shown). The antibodies identified in this study showed affinity to a number of epitopes on HER2, but essentially all bound to the extracellular domain peptides spanning amino acids 328 to 384. Epitopes derived from this region of HER2 have been used by a number of investigators to induce highly effective antibodies to HER2 in animal models. Jasinska et al. (33) used peptides spanning putative B cell epitopes of human HER2 to immunize mice against the human HER2 protein. Of seven peptides, the most immunogenic peptide encompassed amino acids 378 to 398, and antibodies to this peptide effectively suppressed HER2-positive tumor proliferation. Of interest is their finding that the antibodies can mediate complement- and antibody-dependent cell lysis, as well as proliferation suggesting that antibodies to this region might mediate suppressive effects through multiple mechanisms *in vivo*. Dakappagari et al. tested a variety of predicted B cell epitopes from human HER2 fused to an exogenous T-cell epitope, and found that the chimeric peptide spanning amino acids 316 to 339 was the most effective single peptide tested (32). These data suggest that the region of the extracellular domain spanning amino acids 328 to 384

contains multiple epitopes capable of inducing effective antibody immunity to HER2.

Only a subset of patients who were vaccinated developed antibodies, which were primarily of the IgG3 isotype and of relatively low avidity. Despite the low avidity, significant levels of antibodies were induced and were generally highest in those patients whose antibodies were capable of suppressing HER2 phosphorylation. The strategy of combining immunogenic peptides with granulocyte macrophage colony-stimulating factor was done with an aim towards generating Th1 type responses, with subsequent induction of CD8⁺ CTL. In general, vaccine strategies favoring the induction of Th1 responses would be expected to produce peptide-specific CTL and to shift the antibody isotype to IgG2a or IgG3 isotypes (34). In this case, all patients who developed anti-HER2-specific antibodies had evidence of significant T-cell responses with stimulation indices of 2 to 59 (12). Induced IgG concentrations generally did not correlate with avidity. Vaccination with other antigens has been shown to increase target-specific antibody levels while lowering baseline avidity, without compromising efficacy of the vaccine (35). An increase of HER2- and peptide-specific antibody levels ≥ 2 fold was found in all patients who developed antibodies. One potential explanation for the low avidity antibodies found in this study would be that any high avidity antibodies could potentially be cleared from the circulation as immune complexes with soluble HER2 or through binding to HER2 in normal or neoplastic tissues. Given the functional significance of antibodies with specificity to the p328-384, dedicated efforts to generate higher levels of antibody and induction of a Th2 response utilizing B-cell epitope-specific vaccine strategies might be the most appropriate next step to determine whether induction of functional antibodies would be more efficacious (32).

In summary, this study shows that antibodies induced by HER2 peptide vaccination are capable of suppressing HER2 phosphorylation in a site-specific fashion. Suppression of HER2 phosphorylation can also suppress downstream signaling through ERK. In some cases suppression of signaling by these antibodies mediates suppression of the transformed phenotype as tested in colony forming assays. Because the majority of the functional antibodies had specificity to peptide 328 to 345, this epitope is a prime candidate for vaccine targeting. Combined targeting of p328 with other epitopes recognized by tumor inhibitory anti-HER2 antibodies (e.g., p531-586) would be a rational approach to generating functional antibodies in patients with HER2-expressing malignancy.

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References

1. Ben-Levy R, Paterson HF, Marshall CJ, Yarden Y. A single autophosphorylation site confers oncogenicity to the Neu/ErbB-2 receptor and enables coupling to the MAP kinase pathway. *EMBO J* 1994;13:3302-11.
2. Dankort D, Jeyabalan N, Jones N, Dumont DJ, Muller WJ. Multiple ErbB-2/Neu phosphorylation sites mediate transformation through distinct effector proteins. *J Biol Chem* 2001;276:38921-8.
3. Klapper LN, Waterman H, Sela M, Yarden Y. Tumor-inhibitory antibodies to HER-2/ErbB-2 may act by recruiting c-Cbl and enhancing ubiquitination of HER-2. *Cancer Res* 2000;60:3384-8.
4. Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate *in vivo* cytotoxicity against tumor targets. *Nat Med* 2000;6:443-6.
5. Yakes FM, Chinratanalab W, Ritter CA, King W,

- Seelig CS, Arteaga CL. Herceptin-induced inhibition of phosphatidylinositol-3 kinase and Akt is required for antibody-mediated effects on p27, cyclin D1, and antitumor action. *Cancer Res* 2002;62:4132-41.
6. Cuello M, Ettenberg SA, Clark AS, et al. Down-regulation of the erbB-2 receptor by trastuzumab (herceptin) enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in breast and ovarian cancer cell lines that overexpress erbB-2. *Cancer Res* 2001;61:4892-900.
 7. Agus DB, Akita RW, Fox WD, et al. Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth. *Cancer Cell* 2002;2:127-37.
 8. Le XF, Claret FX, Lammayot A, et al. The role of cyclin-dependent kinase inhibitor p27Kip1 in anti-HER2 antibody-induced G1 cell cycle arrest and tumor growth inhibition. *J Biol Chem* 2003;278:23441-50.
 9. Bei R, Masuelli L, Moriconi E, et al. Immune responses to all ErbB family receptors detectable in serum of cancer patients. *Oncogene* 1999;18:1267-75.
 10. Disis ML, Calenoff E, McLaughlin G, et al. Existing T-cell and antibody immunity to HER-2/*neu* protein in patients with breast cancer. *Cancer Res* 1994;54:16-20.
 11. Disis ML, Pupa SM, Gralow JR, Dittadi R, Menard S, Cheever MA. High-titer HER-2/*neu* protein-specific antibody can be detected in patients with early-stage breast cancer. *J Clin Oncol* 1997;15:3363-7.
 12. Disis ML, Gooley TA, Rinn K, et al. Generation of T-cell immunity to the HER-2/*neu* protein after active immunization with HER-2/*neu* peptide-based vaccines. *J Clin Oncol* 2002;20:2624-32.
 13. Disis ML, Goodell V, Schiffman K, Knutson KL. Humoral epitope-spreading following immunization with a HER-2/*neu* peptide based vaccine in cancer patients. *J Clin Immunol* 2004;24:571-8.
 14. Ward RL, Hawkins NJ, Coomber D, Disis ML. Antibody immunity to the HER-2/*neu* oncogenic protein in patients with colorectal cancer. *Hum Immunol* 1999;60:510-5.
 15. Disis ML, Schiffman K, Guthrie K, et al. Effect of dose on immune response in patients vaccinated with an her-2/*neu* intracellular domain protein-based vaccine. *J Clin Oncol* 2004;22:1916-25.
 16. Reilly RT, Machiels JP, Emens LA, et al. The collaboration of both humoral and cellular HER-2/*neu*-targeted immune responses is required for the complete eradication of HER-2/*neu*-expressing tumors. *Cancer Res* 2001;61:880-3.
 17. Piechocki MP, Pilon SA, Wei WZ. Complementary antitumor immunity induced by plasmid DNA encoding secreted and cytoplasmic human ErbB-2. *J Immunol* 2001;167:3367-74.
 18. Renard V, Sonderbye L, Ebbehøj K, et al. HER-2 DNA and protein vaccines containing potent Th cell epitopes induce distinct protective and therapeutic antitumor responses in HER-2 transgenic mice. *J Immunol* 2003;171:1588-95.
 19. Amundadottir LT, Leder P. Signal transduction pathways activated and required for mammary carcinogenesis in response to specific oncogenes. *Oncogene* 1998;16:737-46.
 20. Vijapurkar U, Kim MS, Koland JG. Roles of mitogen-activated protein kinase and phosphoinositide 3'-kinase in ErbB2/ErbB3 coreceptor-mediated heregulin signaling. *Exp Cell Res* 2003;284:291-302.
 21. Timms JF, White SL, O'Hare MJ, et al. Effects of ErbB-2 overexpression on mitogenic signalling and cell cycle progression in human breast luminal epithelial cells oncogenic Ras/Her-2 mediate hyperproliferation of polarized epithelial cells in 3D cultures and rapid tumor growth via the PI3K pathway. *Oncogene* 2002;21:6573-86.
 22. Seton-Rogers SE, Lu Y, Hines LM, et al. Cooperation of the ErbB2 receptor and transforming growth factor β in induction of migration and invasion in mammary epithelial cells. *Proc Natl Acad Sci U S A* 2004;101:1257-62.
 23. Kurokawa H, Lenferink AE, Simpson JF, et al. Inhibition of HER2/*neu* (erbB-2) and mitogen-activated protein kinases enhances tamoxifen action against HER2-overexpressing, tamoxifen-resistant breast cancer cells. *Cancer Res* 2000;60:5887-94.
 24. Oh AS, Lorant LA, Holloway JN, Miller DL, Kern FG, El-Ashry D. Hyperactivation of MAPK induces loss of ER α expression in breast cancer cells. *Mol Endocrinol* 2001;15:1344-59.
 25. Yip YL, Novotny J, Edwards M, Ward RL. Structural analysis of the ErbB-2 receptor using monoclonal antibodies: Implications for receptor signalling. *Int J Cancer* 2003;104:303-9.
 26. Stancovski I, Hurwitz E, Leitner O, Ullrich A, Yarden Y, Sela M. Mechanistic aspects of the opposing effects of monoclonal antibodies to the ERBB2 receptor on tumor growth. *Proc Natl Acad Sci U S A* 1991;88:8691-5.
 27. Pinkas-Kramarski R, Soussan L, Waterman H, et al. Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. *EMBO J* 1996;15: 2452-67.
 28. Cobleigh MA, Vogel CL, Tripathy D, et al. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J Clin Oncol* 1999;17:2639-48.
 29. Sliwkowski MX, Lofgren JA, Lewis GD, Hotelling TE, Fendly BM, Fox JA. Nonclinical studies addressing the mechanism of action of trastuzumab (Herceptin). *Semin Oncol* 1999;26:60-70.
 30. De Santes K, Slamon D, Anderson SK, et al. Radiolabeled antibody targeting of the HER-2/*neu* oncoprotein. *Cancer Res* 1992;52:1916-23.
 31. Motoyama AB, Hynes NE, Lane HA. The efficacy of ErbB receptor-targeted anticancer therapeutics is influenced by the availability of epidermal growth factor-related peptides. *Cancer Res* 2002; 62:3151-8.
 32. Dakappagari NK, Pyles J, Parihar R, Carson WE, Young DC, Kaumaya PT. A chimeric multi-human epidermal growth factor receptor-2 B cell epitope peptide vaccine mediates superior antitumor responses. *J Immunol* 2003;170:4242-53.
 33. Jasinska J, Wagner S, Radauer C, et al. Inhibition of tumor cell growth by antibodies induced after vaccination with peptides derived from the extracellular domain of Her-2/*neu*. *Int J Cancer* 2003;107: 976-83.
 34. Rizzo LV, DeKruyff RH, Umetsu DT. Generation of B cell memory and affinity maturation. Induction with Th1 and Th2 T cell clones. *J Immunol* 1992;148:3733-9.
 35. Spoulou V, Victoratos P, Ioannidis JP, Grafakos S. Kinetics of antibody concentration and avidity for the assessment of immune response to pneumococcal vaccine among children with bone marrow transplants. *J Infect Dis* 2000;182:965-9.