

Prevention of Mammary Tumors with a Chimeric HER-2 B-cell Epitope Peptide Vaccine¹

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ABSTRACT

Synthetic peptide vaccines targeting B-cell epitopes of the extracellular domain of the HER-2 oncoprotein were evaluated for their capacity to elicit HER-2-specific antibodies with antiproliferative activity. Several HER-2 B-cell epitopes were identified by computer-aided analysis of protein antigenicity, and selected B-cell epitopes were synthesized colinearly with a promiscuous T-helper epitope (208–302) derived from the measles virus fusion protein at either the NH₂ or COOH terminus linked via a four-residue turn sequence (GPSL). In addition, one epitope sequence, 628–647, was mutated to optimize disulfide pairing to mimic the native HER-2 receptor. All of the four selected epitopes elicited high-titered antibodies in outbred rabbits with exceptionally high titers for MVF-HER-2(628–647). These antibodies were cross-reactive with the native HER-2 receptor. Antibodies elicited by MVF-HER-2(628–647) inhibited proliferation of human HER-2-overexpressing breast cancer cells *in vitro* and caused their antibody-dependent cell-mediated cytotoxicity. Furthermore, immunization with MVF-HER-2(628–647) prevented the spontaneous development of HER-2/neu-overexpressing mammary tumors in 83% of transgenic mice. The engineered, chimeric peptide B-cell immunogen MVF-HER-2(628–647) may have applications in the prevention of HER-2-overexpressing cancers.

INTRODUCTION

HER-2 is a M_r 185,000 transmembrane phosphoglycoprotein encoded by the *erbB-2* gene, the human homologue of the rat proto-oncogene *neu*. HER-2 is a member of the EGFR⁴ (EGFR/*erbB-1*) family. It is composed of an ECD that is cysteine rich and has several glycosylation sites and an intracellular domain with a highly conserved tyrosine kinase (1, 2). Although a direct ligand for HER-2 has not been described, it has been shown to function as a preferential heterodimerization signaling partner with EGFR, HER-3, and HER-4 by providing a low-affinity ligand binding site (3, 4). In humans, HER-2 is expressed in fetal tissues and at low levels in normal tissues of adults (5). Overexpression of HER-2 is associated with 20–30% of breast and ovarian cancers and, to a lesser extent, with adenocarcinoma of uterus, cervix, fallopian tube, and endometrium (6–8). In patients with breast cancer, HER-2 overexpression is an independent predictor of survival; it is associated with poor prognosis, aggressive disease, and resistance to chemotherapy and hormone therapy (8–10). How HER-2 alters the growth of normal or cancer cells is not entirely clear. HER-2 overexpression may provide tumors with a selective growth advantage through increased utilization of stromal-derived

epidermal growth factor-like growth factors or ligand-independent receptor homodimerization (11, 12).

HER-2 is an attractive target for immunotherapeutic approaches. Antibodies directed against the ECD of HER-2 have been shown to confer inhibitory effects on tumor growth *in vitro* and in animal models (13–18). In Phase II and Phase III clinical trials, a recombinant humanized anti-HER-2 monoclonal antibody, Trastuzumab, produced an overall response rate of 15% as a single agent in patients with metastatic HER-2-overexpressing breast cancers and has been shown to improve survival when combined with cytotoxic chemotherapeutics (19–21). The molecular mechanisms underlying these growth-inhibitory effects are not well understood. Initial studies showed that antibodies to HER-2 could cause receptor internalization and degradation with reduced phosphorylation resulting in the inhibition of tumor cell growth (14, 22, 23). There is evidence that HER-2 antibodies can block heterodimer formation, interfere with ligand binding, or trigger apoptosis (24–26). HER-2 antibodies also mediate complement-dependent cytotoxicity and/or ADCC (27–29).

Active specific immunotherapy offers the possibility of generating sustained anti-HER-2 immune responses and is potentially more effective than passive approaches, particularly when the application is primary or secondary cancer prevention. A number of vaccine approaches targeting p185 HER-2 or the HER-2 ECD have been evaluated. Strain NFS mice immunized with a vaccinia virus recombinant that expresses the ECD rat *neu* developed a protective antibody response against subsequent challenge with *neu*-transformed NIH 3T3 cells (30). However, immunization of BDIX rats with the same immunogen did not result in antibody response, nor did it inhibit the growth of syngeneic *neu*-expressing B104 neuroblastoma cells, suggesting that this strategy was insufficient to induce immune responses in the rat. A polysaccharide-oncoprotein complex vaccine consisting of the 147 NH₂-terminal amino acids of HER-2 ECD complexed with cholesteryl group-bearing mannan and pullulan induced cellular and humoral immune responses that mediated rejection of HER-2-expressing sarcomas in BALB/c mice (31). Partial protection was shown in rat *neu* transgenic mice destined to develop mammary tumors by immunizing them with either a purified rat *neu* ECD (32) or *neu*-transfected allogeneic mouse fibroblasts (33).

Despite the evidence presented above, it is not entirely clear whether effective immune responses can be generated in humans using cell- or protein-based vaccine strategies targeting p185 HER-2 or the HER-2 ECD because HER-2 is a nonmutated “self” antigen. Moreover, some antibodies elicited to HER-2 have been shown to stimulate rather than inhibit the growth of human tumors, and HER-2 vaccines presenting multiple epitopes could potentially elicit a mixture of counterproductive humoral responses (22). Immunization to self tumor antigens may require a vaccine design that targets a portion of the protein rather than whole protein domains of the antigen. There may be advantages to the use of subunit peptide-based immunogens when targeting HER-2 not only to elicit a desired immune response but also to circumvent tolerance to native protein. Disis *et al.* (34) have shown that immunization of rats with multiple T-helper peptides derived from the rat *neu* protein elicited strong humoral and CD4+ responses; in contrast, immunization with purified whole rat *neu*

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⁴ The abbreviations used are: EGFR, epidermal growth factor receptor; ECD, extracellular domain; ADCC, antibody-dependent cell-mediated cytotoxicity; MVF, measles virus fusion epitope sequence 208–302; PBMC, peripheral blood mononuclear cell; PBT, PBS containing 0.05% Tween 20 and 1% horse serum.

protein in parallel experiments failed to elicit detectable immune responses. Recently, these investigators also showed that immunization of breast and ovarian cancer patients with multiple HER-2 peptides selected for binding to MHC class II molecules elicited both peptide- and protein-specific T-helper cell responses (35). Whether immune responses elicited by peptide immunogens incorporating human HER-2 T-helper cell epitopes will be of sufficient potency to mediate antitumor activity in humans is not known. The genetic MHC-restricted stimulatory activity of human self-peptides corresponding to T-cell epitopes is also a major obstacle to developing T-cell peptide vaccine approaches for use in an "outbred" human population.

We hypothesized that a rationally designed peptide vaccine targeting specific B-cell determinants from the HER-2 ECD could induce antibodies capable of inhibiting the growth of HER-2-expressing cancers. To augment antibody responses and overcome MHC genetic polymorphism, "promiscuous" T-helper peptide epitopes from a non-human molecule may be incorporated. The B-cell HER-2 epitopes were designed with a minimal number of point mutations to facilitate folding of the peptide into a stable conformation to mimic the native protein structure. They were synthesized colinearly with a promiscuous T-helper cell epitope derived from amino acid sequence 288–302 of the measles virus fusion protein. MVF has previously been shown to interact with several distinct human MHC class II alleles (36). Furthermore, we have shown that MVF-conjugated B-cell epitope peptide constructs could be used to bypass certain haplotype-restricted immune responses and provide broad immunogenicity in a large number of individuals typical of an outbred population (37–42). Here we demonstrate that chimeric peptide immunogens targeting a single HER-2 B-cell epitope and incorporating a promiscuous T-helper epitope are capable of eliciting high-titered, native receptor-specific humoral responses in outbred rabbits. Antibodies elicited by one of these immunogens, MVF HER-2(628–647), could selectively inhibit the growth of HER-2-overexpressing cells. Moreover, active immunization with this peptide construct prevented the development of tumors in a transgenic mouse model of HER-2/neu mammary tumorigenesis.

MATERIALS AND METHODS

B-cell Epitope Prediction and Peptide Synthesis. The selection of candidate B-cell epitopes expressed within the human HER-2 ECD was accomplished by computer-aided analysis using various correlates of protein antigenicity as reviewed by Kaumaya *et al.* (43). The basic premise is that algorithms used in this analysis will always locate regions that are surface-exposed on the protein and therefore most likely to be involved in antibody binding. Selected B-cell epitopes were synthesized colinearly with the T-helper epitope MVF using a 4-residue amino acid linker (GPSL) as described previously (44) either on a Milligen/Biosearch 9600 peptide synthesizer (Bedford, MA) or a multiple peptide synthesizer (Model 396; Advance Chemtech, Louisville, KY) using a 4-methylbenzhydrylamine resin as the solid support (substitution, 0.54mm/g). The Fmoc/t-butyl synthetic method was used, using 4-(hydroxymethyl) phenoxyacetic acid as the linker. After the final deprotection step, protecting groups and peptide resin bond were cleaved with 90% trifluoroacetic acid, 5% anisole, 3% thioanisole, and 2% ethanedithiol. The

crude peptides were purified by reverse-phase high-performance liquid chromatography and were >95% pure before immunization. The identity of the peptides was confirmed by krotos IV MALDI-TOF matrix-assisted laser desorption ionization-time of flight spectrometry at the Complex Carbohydrate Research Center (Athens, GA).

Immunization of Rabbits and Transgenic Mice. Female New Zealand White rabbits were obtained from Mohican Valley Rabbitry (Loudenville, OH). Pairs of rabbits were immunized s.c. at multiple sites with a total of 1 mg of each of the four chimeric peptides (Table 1) emulsified in complete Freund's adjuvant. Subsequent booster injections, 1 mg and 500 μ g of the peptide in PBS, were given 3 and 6 weeks after the primary immunization. Sera were collected, and complement was inactivated by heating to 56°C for 30 min. High-titered sera were purified on a protein A/G-agarose column (Pierce, Rockford, IL), and eluted antibodies were concentrated and exchanged in PBS using M_r 100,000 cutoff centrifuge filter units (Millipore, Bedford, MA). The concentration of antibodies was determined by the Coomassie plus protein assay reagent kit (Pierce). Transgenic mice (strain N202) overexpressing the rat *neu* gene under the transcriptional control of the mouse mammary tumor virus promoter were purchased from The Jackson Laboratory (Bar Harbor, ME). Groups of six transgenic mice, each 4–6 weeks old, were immunized separately with 100 μ g of HER-2(115–136) MVF, HER-2(410–429) MVF, and MVF HER-2(628–647). The peptides were dissolved in PBS with 100 μ g of muramyl dipeptide adjuvant *N*-acetyl-glucosamine-3 γ -L-acetyl-L-alanyl-D-isoglutamine and emulsified (50:50) in Squalene/Arlacel A oil (4:1) as described elsewhere (45). Nine mice were injected with MVF/*N*-acetyl-glucosamine-3 γ -L-acetyl-L-alanyl-D-isoglutamine emulsion as immunized controls. Boosters were given s.c. after 4, 8, 16, and 24 weeks. Two more boosters were also given at 32 and 40 weeks with only MVF HER-2(628–647) to sustain the high-titered immune responses. Mice were retro-orbitally bled monthly for antibody titer determination. Tumor size (length and width) was measured with vernier calipers. Individual tumor volumes were calculated by the formula (length \times width²/2).

ELISA. The 96-well plates were coated with 100 μ l of antigen at 2 μ g/ml in PBS overnight at 4°C. Nonspecific binding sites were blocked for 1 h with 200 μ l of PBS-1% BSA, and plates were washed with PBT. Rabbit antiserum (1:500) or mouse antiserum (1:50) in PBT was added to antigen-coated plates in duplicate wells, serially diluted 1:2 in PBT, and incubated for 2 h at room temperature. After washing the plates, 100 μ l of 1:500 goat antirabbit or goat antimouse IgG conjugated to horseradish peroxidase (Pierce) were added to each well and incubated for 1 h. After washing, the bound antibody was detected using 50 μ l of 0.15% H₂O₂ in 24 mM citric acid and 5 mM sodium phosphate buffer (pH 5.2) with 0.5 mg/ml 2,2'-aminobis(3-ethylbenzthiazoline-6-sulfonic acid) as the chromophore. Color development was allowed to proceed for 10 min, and the reaction was stopped with 25 μ l of 1% SDS. Absorbance was determined at 410 nm using a Dynatech MR700 ELISA reader (Chantilly, VA). Titers were defined as the highest dilution of sera with an absorbance of greater than 0.2 after subtracting the background.

Mouse Isotyping. MVF HER-2(628–647) antibodies raised in transgenic mice were typed using a Mouse Typer Sub-Isotyping Kit (Bio-Rad, Hercules, CA). The assay was performed according to the manufacturer's instructions, except that a 1:1000 dilution of goat antirabbit IgG horseradish peroxidase conjugate was used.

Cell Culture. All cell culture media, FCS, and supplements were purchased from Life Technologies, Inc. (Grand Island, NY). The human breast adenocarcinoma cell lines SK-BR-3 and BT-474 overexpressing HER-2 and the rat *neu*-overexpressing fibroblast cell line DHFR-G8 were purchased from American Type Culture Collection (Manassas, VA) and maintained according to the suppliers' guidelines. CAV-1 was derived from a fresh colon tumor

Table 1 Amino acid sequences and the structural attributes of chimeric B-cell epitope constructs used in this work

MVF sequence is italicized. *N*-linked glycosylation sites are shown in bold. The cysteine to glycine mutation in HER-2(628–647) is underlined.

B-cell epitope constructs	Amino acid sequence	Predicted secondary structures	M_r
HER-2(115–136) MVF	H ₂ N-AVLDNGDPL LNNTT PVTGASPGG-GPSL-KLLSLIKGVIVHRLEGVE-COOH	117–119 turn; 123–129 β sheet	4407
HER-2(376–395) MVF	H ₂ N-FLPESFDGDPASNTAPLQPE-GPSL-KLLSLIKGVIVHRLEGVE-COOH	376–379 & 389–395 α -helix; 382–384 turn	4472
HER-2(410–429) MVF	H ₂ N-LYISAWPDSL PDLSV FQNLQ-GPSL-KLLSLIKGVIVHRLEGVE-COOH	421–429 β sheet	4646
MVF HER-2(628–647)	H ₂ N-KLLSLIKGVIVHRLEGVE- <u>LSPG</u> - INGTH SCVDLDDKGCPEQR-COOH	632–634 β sheet; 635–637 & 642–646 α helix; 638–639 turn	4498

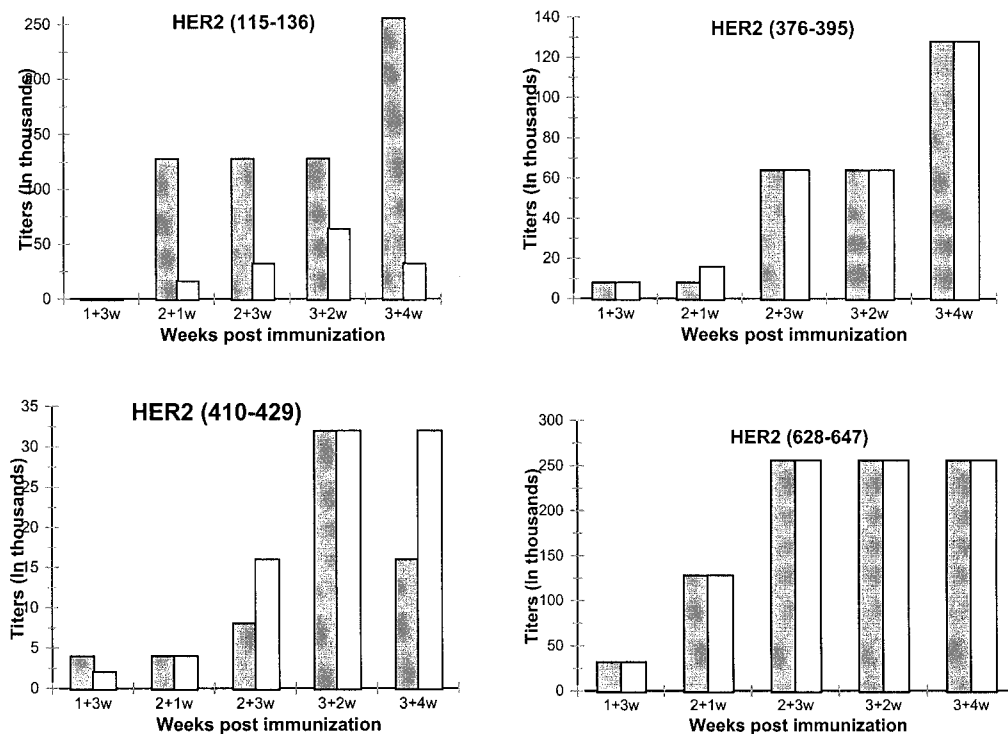


Fig. 1. Immune responses to HER-2 peptide constructs (indicated at the top of each panel) in two different rabbits (■ and □) were determined by ELISA. Designation is shown on the X axis (e.g., 1+3w on the X axis indicates sera collected 3 weeks after the first immunization).

specimen that was cryopreserved and subsequently cultured. This cell line does not express detectable levels of HER-2/neu. CAV-1 was maintained in RPMI 1640 with 10% FCS and L-glutamine.

Immunoprecipitation and Western Blotting. SK-BR-3 or DHFR-G8 cells (1×10^7) suspended in 100 μ l of HBSS per sample were lysed in 1 ml of ice-cold 0.5% NP40 lysis buffer [150 mM NaCl, 50 mM Tris (pH 8), 10 mM EDTA, 10 mM Na₂PP₆, 10 mM sodium fluoride, 1% NP40, and 0.1% SDS] containing 10 μ g/ml each of aprotinin and leupeptin. Lysis was achieved by gentle rotation at 4°C for 20 min. After centrifugation (14,000 \times g, 10 min) to remove cell debris, lysates were incubated with 10 μ g of anti-peptide antibody and 30 μ l of protein A/protein G (Calbiochem, La Jolla, CA) overnight. Beads were pelleted by centrifugation (14,000 \times g 30 s), washed twice in lysis buffer containing 1 mM Na₃VO₄, and boiled in SDS sample buffer for 3 min. Proteins were resolved by 7.5% SDS-PAGE, transferred to nitrocellulose, and then probed with HER-2- or rat neu-specific monoclonal antibodies (Calbiochem). Protein transfer was monitored with prestained molecular weight standards (Bio-Rad). Immunoreactive bands were detected by enhanced chemiluminescence (Pierce) using horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins.

Flow Cytometry. This procedure was adopted from that described by Hudziak *et al.* (13). Briefly, 5×10^5 SK-BR-3 or DHFR-G8 cells were incubated with either 2.5 μ g of rabbit anti-peptide antibodies or a 1:40 mouse sera dilution and HER-2-specific mouse monoclonal antibody Ab-2 and rat neu-specific monoclonal antibody Ab-4 (Calbiochem) were used as positive controls, and isotypic IgG was used as a negative control for 1 h at 4°C in 100 μ l of PBS/1% FCS. The cells were washed twice in PBS and incubated with FITC-labeled secondary antibody (1:50 dilution) for 30 min at 4°C in 100 μ l of PBS/1% FCS. The cells were washed twice, fixed in 2% formaldehyde, and analyzed by a Coulter ELITE flow cytometer (Coulter, Hialeah, FL). A total of 10,000 cells were counted for each sample, and final processing was performed. Debris, cell clusters, and dead cells were gated out by light scattered assessment before single-parameter histograms were drawn and smoothed.

Cell Proliferation Assay. SK-BR-3 and CAV-1 cells were plated at 5000 cells/well in V-bottomed plates with anti-peptide antibodies at 10 μ g/ml on day 0. On day 3, cells were pulsed with [³H]thymidine (1 μ Ci/well) for 6 h and then placed in a -20°C freezer for 1 h. After thawing at room temperature, cells were harvested using a PHD cell harvester. Samples were incubated in 5

ml of Ready Safe liquid scintillation mixture (Beckman, Fullerton, CA), and radioactivity was determined by using a beta counter. Results are expressed as the percentage of inhibition [(untreated - treated)/untreated \times 100] of triplicate samples.

ADCC Assay. PBMCs were isolated from heparinized whole blood obtained from normal human donors by density gradient sedimentation using Ficoll-Hypaque (Pharmacia Biotech, Piscataway, NJ). The purified PBMCs were washed twice with culture medium (RPMI 1640-1% FCS) and serially diluted into 96-well plates to give E:T ratios of 50:1, 25:1, 12.5:1, or 6.25:1. Protein A/G purified Her-2(628-647) peptide antibodies from immunized transgenic mice and the clinically applied HER-2 monoclonal antibody Trastuzumab (Genentech Inc, South San Francisco, CA) were added at 2 μ g/well. Target cell lines (SK-BR-3 or BT-474) were labeled with 200 μ Ci of Na⁵¹CrO₄ (New England Nuclear Life Science Products, Boston, MA) by incubating them for 45 min in a CO₂ humidified chamber at 37°C and washed three times in the cultured medium. A total of 0.1 ml of target cells (10⁵/ml) was added per well for a final volume of 0.2 ml/well. Target cells were incubated with PBMCs in absence of antibodies to assess nonspecific lysis. The plates were incubated for 4 h at 37°C, and then the supernatants were harvested, and the radioactivity was determined using a gamma counter. The percentage of lysis or cytotoxicity was calculated as follows: Cytotoxicity (%) = $(A - B/C - B) \times 100$, where A represents ⁵¹Cr (cpm) from test supernatants, B represents spontaneous release (⁵¹Cr from target cells without antibody treatment), and C represents maximum release (⁵¹Cr from target cells lysed with 5% Triton-X114). Each treatment was performed in triplicate and averaged before calculating the percentage of lysis.

RESULTS

Chimeric HER-2 B-cell Epitope Constructs. Four of the 12 highest scoring (of the 144 analyzed) HER-2 ECD B-cell epitope sequences, amino acid sequences 115-136, 376-395, 410-429, and 628-647, were selected for evaluation (Table 1). Amino acid sequence alignment indicated that epitope 115-136 is highly variable between EGFR, HER-2, HER-3, and HER-4. Therefore, this sequence was hypothesized to have some unique function in HER-2 such as

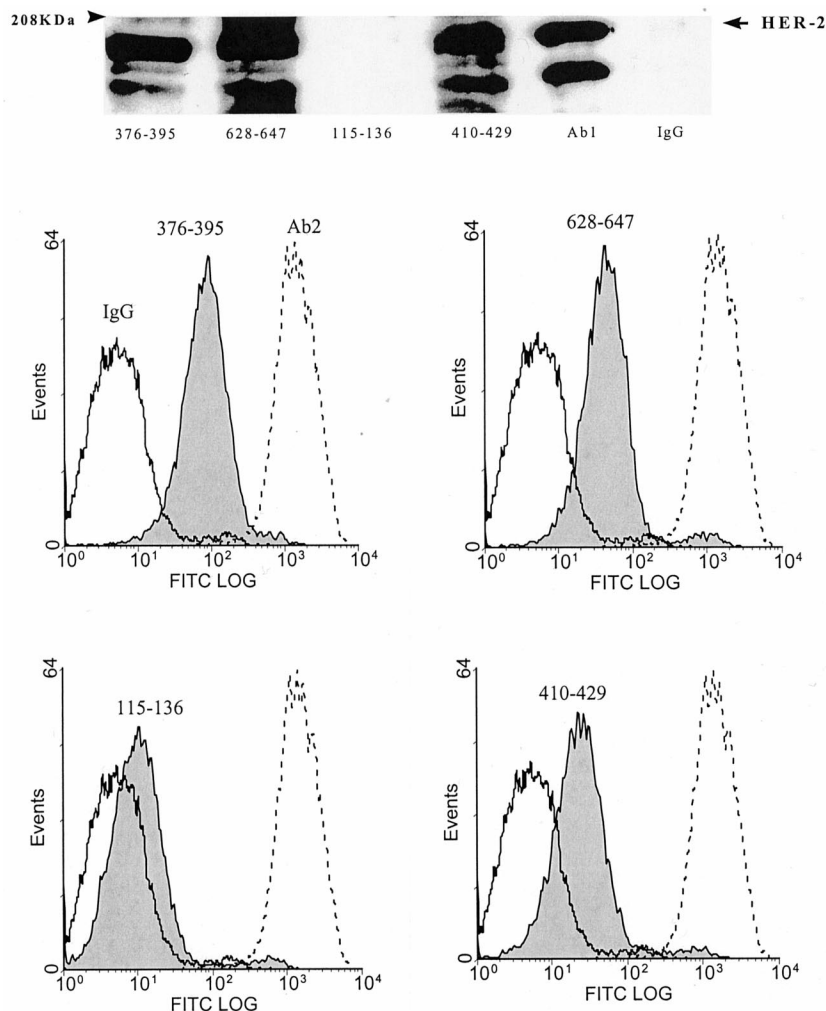


Fig. 2. Binding of peptide antibodies to the HER-2 receptor was determined by immunoprecipitation (*top*) and flow cytometry (*bottom*) using SB-BR-3 cells. IgG is an isotype antibody control. Ab-1 is a HER-2-specific monoclonal antibody used as a positive control for immunoprecipitation and Western blotting. Ab-2 is a mouse monoclonal antibody specific to the HER-2 ECD used as a positive control in flow cytometry.

ligand binding. Antibodies raised to this region were hypothesized to inhibit tumor growth by blocking HER-2 receptor signaling. Epitope 628–647 was chosen because of its proximity to the cell membrane. Antibodies binding to the juxtamembrane region were hypothesized to cause receptor aggregation and perturb the cell membrane more effectively, leading to HER-2 receptor endocytosis and degradation. Epitope sequences 376–395 and 410–429 were chosen because of their relative immunogenic potential, based on our predictive rankings. Neither of these two sequences contained cysteines or potential *N*-linked glycosylation sites, and these two sequences were predicted to form one secondary structural element, either an α -helix (sequence 376–395) or a β -sheet (sequence 410–429). HER-2 sequences 115–136, 376–395, and 410–429 were synthesized with the promiscuous T-helper cell epitope, MVF, at the COOH terminus, and sequence 628–647 was synthesized with MVF at the NH₂ terminus. The orientation of the T-helper cell epitope was chosen based on sequence-dependent difficulties for assembly of the peptide. However, the orientation of MVF does not affect the immunogenicity of the peptide constructs (46). These chimeric peptides incorporate a 4-residue linker (GPSL), in which glycine and proline in the linker potentiate a β turn in the oligopeptide, whereas serine in that position will favor hydrogen bonds with the free NH of the backbone. Leucine in the sequence was chosen because its side chain in that position is completely buried in the hydrophobic core and must be hydrophobic. The flexible nature of the linker allows for independent folding of the T-helper cell and B-cell epitopes (43, 46). HER-2 sequence 628–647

contains three cysteines whose disulfide bond pairing was unknown. Cys-634 and Cys-642 were hypothesized to form a suitable disulfide bridge, based on their proximity and predicted secondary structure. Thus, Cys-630 was mutated to glycine because the relatively small size of the R group of glycine causes minimal steric hindrance to formation of predicted β -sheet structure. Sequences 115–136 and 628–647 have potential *N*-linked glycosylation sites 124-NNTT-127 and 629-NCTH-632 respectively; however, the latter site is a poor sugar acceptor due to steric hindrance caused by the propensity of cysteine to form disulfide bonds (47). The crude peptides were purified by reverse-phase high-performance liquid chromatography and were >95% pure before immunization. The identity of the peptides was confirmed by mass spectrometry. The amino acid sequences, predicted secondary structures, posttranslational modifications, and the molecular weights of the MVF-conjugated HER-2 peptide constructs are indicated in Table 1.

Immunogenicity of Chimeric HER-2 B-cell Epitope Peptides in Outbred Rabbits. The HER-2 oligopeptides were highly immunogenic, as evidenced by antibody titers of over 100,000 (Fig. 1). HER-2(115–136) MVF elicited immediate and high antibody titers 1 week after the first booster in one of the two rabbits; however, the antibody response to this construct rose slowly in the other rabbit to high titers by 2 weeks after the second booster. The HER-2(376–395) MVF immune response was characterized by a slightly longer lag phase with an eventual rise in antibody titers to maximal levels after the tertiary boost. The antibody response to HER-2(410–29) MVF

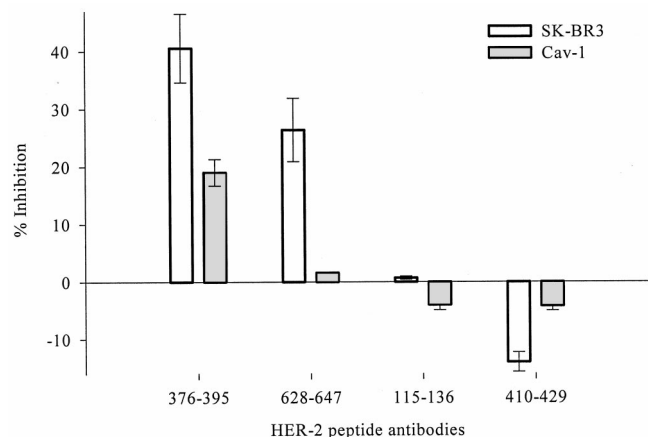


Fig. 3. Growth-inhibitory effects of peptide antibodies on SK-BR-3 and CAV-1 cells were determined by standard [^3H]thymidine proliferation assay. Results are expressed as the percentage of inhibition [untreated - treated/untreated \times 100] of averaged triplicate samples. SDs are indicated by error bars.

was relatively low in both rabbits with maximum titers approaching 30,000 within 2 weeks after the tertiary boost. MVF HER-2(628–647) produced the most immediate and vigorous response, with exceptionally high titers of over 250,000 that remained at maximal levels in both rabbits through 4 weeks after tertiary boost. The polyclonal IgG sera did not cross-react with the MVF T-cell sequence.

Binding of Peptide Antibodies to Native HER-2 Receptor. Peptide-based vaccines will be effective only if the antibodies elicited by peptide immunogens bind the HER-2 receptor. Three different methods were used to test the binding of anti-peptide antibodies to the native protein. First, the capacity of HER-2 peptide antibodies to immunoprecipitate HER-2 protein from the lysates of SK-BR-3 cells, a human breast cancer cell line overexpressing HER-2, was assessed. All of the peptide antibodies, except antibodies to HER-2(115–136) MVF, efficiently immunoprecipitated the native receptor from SK-BR-3 cells (Fig. 2). The lower band in the doublet is probably an isoform or underglycosylated counterpart due to high-level expression of HER-2 in SK-BR3 cells and has been observed by others (13, 34). Second, binding of the peptide antibodies to the intact HER-2 receptor was determined by immunofluorescence staining of single cell suspension of SK-BR-3 cells. Antibodies generated against HER-2(376–395) and HER-2(628–647) bound the receptor well within 1 log of HER-2-specific mouse monoclonal antibody Ab-2 (Fig. 2). However, antibodies to HER-2(410–429) showed weak binding and might be related to low titers elicited by rabbits against this immunogen (Fig. 1), whereas antibodies to HER-2(115–136) did not bind the receptor, confirming the immunoprecipitation results. Third, the binding of the peptide antibodies to a recombinant glycosylated HER-2 ECD was determined by an indirect ELISA. The pattern of reactivity of peptide antibodies with the protein paralleled that observed with immunoprecipitation and immunofluorescence staining (data not shown).

Effect on Breast Cancer Cell Proliferation. The effect of the peptide antibodies on tumor growth was examined *in vitro* with a standard [^3H]thymidine proliferation assay using HER-2-overexpress-

ing SK-BR-3 cells and CAV-1, a colon cancer cell line that expresses low levels of HER-2 as a control. Antibodies elicited by HER-2(376–395) MVF and MVF HER-2(628–647) were able to reduce the proliferation of SK-BR-3 cells by about 40% and 30%, respectively, compared with untreated cells (Fig. 3). In contrast, slight increases in proliferation were observed with the antibodies elicited by HER-2(410–429) MVF. The anti-peptide antibodies [with the exception of anti-HER-2(376–395) MVF] had minimal effects on CAV-1 cells, suggesting the possibility that peptide antibodies against MVF HER-2(628–647) selectively modulate the growth of HER-2-overexpressing tumor cell lines.

Effects of MVF-HER-2 Peptide Constructs in Rat *neu* Transgenic Mice. A transgenic mouse model (designated N202) developed by Guy *et al.* (48) that expresses mammary tumors similar to human breast cancer was used to test *in vivo* antitumor effects. Focal mammary tumors arise in at least 50% of the female transgenic mice around 28 weeks of age due to overexpression of the rat *neu* gene under the transcriptional control of the murine mammary tumor virus 3' long terminal repeat. Three of the HER-2 peptide sequences (376–395, 410–429, and 628–647) have >80% homology to the analogous regions in rat *neu* (2). We examined whether the antibodies raised against the HER-2 peptides were capable of recognizing the rat *neu* receptor because there was a 20% amino acid sequence disparity. As depicted in Fig. 4, antibodies elicited with HER-2 sequences 115–136, 410–429, and 628–647 were able to immunoprecipitate the rat *neu* receptor from the *neu* gene-overexpressing DHFR-G8 fibroblast cell line.

Based on these results, female transgenic mice were immunized with HER-2(115–136) MVF, HER-2(410–429) MVF, and MVF HER-2(628–647), or MVF. MVF HER-2(628–647) elicited high-titered antibody responses against the immunogen of over 50,000 as early as 2 weeks after the second booster, and the antibody titers reached more than 250,000 after the third booster (Fig. 5). Antibodies against MVF HER-2(628–647) also reacted with recombinant HER-2 ECD with titers over 10,000 (Fig. 5) and the intact HER-2 and rat *neu* receptors of cells (data not shown). The transgenic mice did not mount appreciable antibody responses against immunogens HER-2(115–136) MVF and HER-2(410–429) MVF. Antibody titers against both these immunogens were below 4000 even 6 weeks after the fourth booster (data not shown).

By 48 weeks of age, all of the transgenic mice immunized with MVF emulsion, HER-2(115–136) MVF and HER-2(410–429) MVF, developed tumors of at least 10 millimeters in size. Most notably, in correlation with the *in vitro* inhibition of tumor cell proliferation (Fig. 3), 83% (five of six) transgenic mice immunized with MVF HER-2(628–647) were completely free of tumors (Fig. 6). MVF HER-2(628–647)-vaccinated mice showed a significantly longer tumor-free interval compared with mice immunized with MVF emulsion ($P = 0.0025$). No tumors were detectable in these mice during the 52 weeks of observation. Although there was a delay in the onset of tumors in the one mouse immunized with MVF HER-2(628–647) compared to other groups there was no significant difference in the kinetics of tumor growth after their occurrence (data not shown).

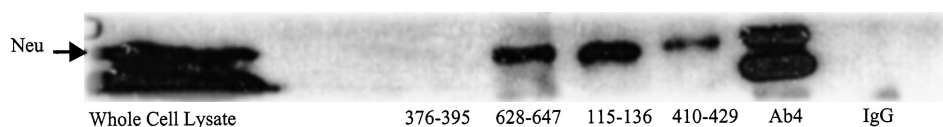


Fig. 4. Cross-reactivity of HER-2 peptide antibodies with rat *neu* receptor was determined by immunoprecipitation from the *neu* gene-overexpressing cell line DHFR-G8. Ab-4 is a monoclonal antibody specific to rat *neu*, and IgG is an isotype antibody control. The protein bands migrating just below the M_r 208,000 marker are shown by Western blotting with Ab-1, which also recognizes rat *neu*.

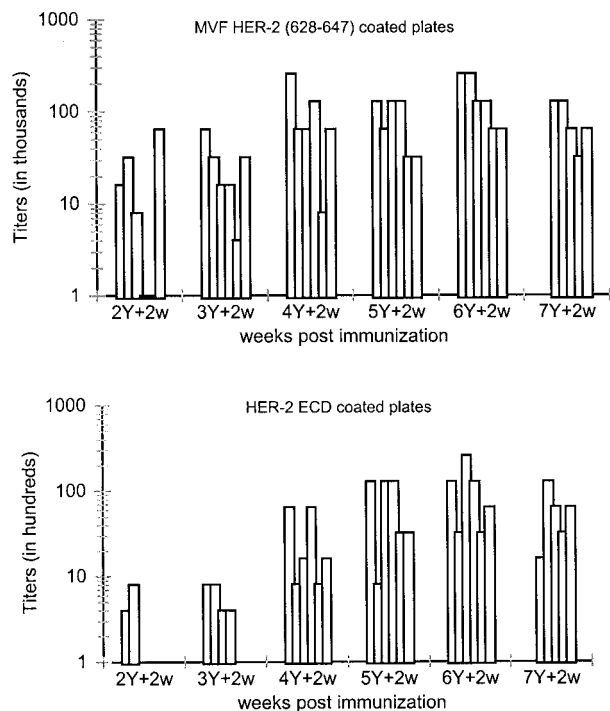


Fig. 5. Immune responses to MVF HER-2(628–647) in six transgenic mice (represented by individual bars) were determined by titrating the sera against the corresponding immunogen (*top*) and glycosylated recombinant HER-2 ECD (*bottom*) by an indirect ELISA.

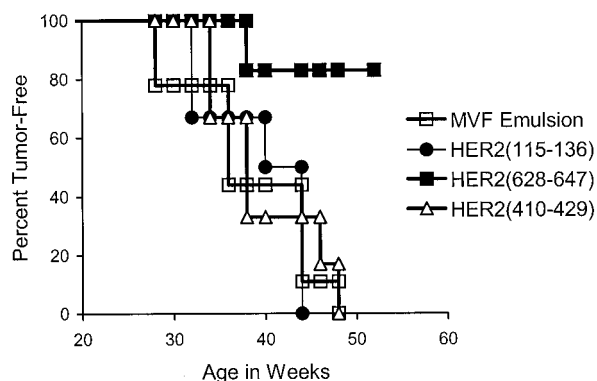


Fig. 6. Immunoprotective effects of HER-2 peptide epitopes on spontaneous tumor development in transgenic mice were determined by immunizing groups of six to nine mice with indicated peptides at 4 weeks of age and boosting every 4 or 8 weeks as described in “Materials and Methods.” Tumor volumes were calculated as $(\text{length} \times \text{width}^2)/2$. The time to tumor development was analyzed using Kaplan-Meier survival analysis with log-rank comparisons of individual curves (62, 63).

Antibody-mediated Cytotoxicity of Breast Tumor Cell lines.

We found IgG1 (58%) and IgG2 (35%) to be the major isotypes in the transgenic mouse sera elicited by MVF HER-2(628–647). HER-2 monoclonal antibodies representing these two isotypes were shown to be capable of mediating ADCC in conjunction with human PBMCs (22, 29). In an attempt to explore the molecular mechanism of tumor growth inhibition by the HER-2(628–647) peptide antibody, we tested its potential to recruit PBMCs to lyse HER-2-overexpressing mammary tumor cell lines in an ADCC assay. Peptide antibodies elicited in transgenic mice by HER-2(628–647) invoked lysis of two different human breast tumor cell lines, SK-BR-3 and BT-474, expressing high levels of HER-2 (27) in presence of human PBMCs, similar to the clinically applied HER-2 monoclonal antibody, Trastuzumab (Fig. 7).

DISCUSSION

The use of peptide immunogens in humans is considered problematic because they have been historically considered to be weak immunogens. Antibodies elicited in animals by immunization with synthetic peptides have generally been shown to have low affinity to the native protein, partly because antibody recognition sites are usually of the conformational type, and the peptide immunogens lacked defined structure in solution. Peptides must mimic the native conformation of the protein for their respective antibodies to bind target antigens with an affinity high enough to be biologically significant. The genetically restricted stimulatory activity of peptides is also a major obstacle to developing vaccine approaches for use in an outbred human population (49). Covalent conjugation of B-cell epitope peptides to large carrier molecules is sometimes used to address this problem but often results in hypersensitivity, conformational changes, appearance of undefined structures, loss of epitopes, inappropriate presentation of epitopes, and batch-to-batch conjugate variability. We have addressed several of these issues in our approach to subunit peptide vaccine design. Our strategy involved *de novo* design of topographic determinants that focused on preserving the native protein sequence while introducing a minimal number of rational point mutations to facilitate folding of the peptide into a stable conformation that mimics the native protein structure (50, 51). We have examined the effectiveness of incorporating promiscuous T-helper epitopes derived from nonhuman molecules into these constructs to overcome human MHC genetic polymorphism (41). Our previous work in a variety of model systems has demonstrated that this approach can elicit high-titered antibodies that recognize native protein in an outbred population (37–42).

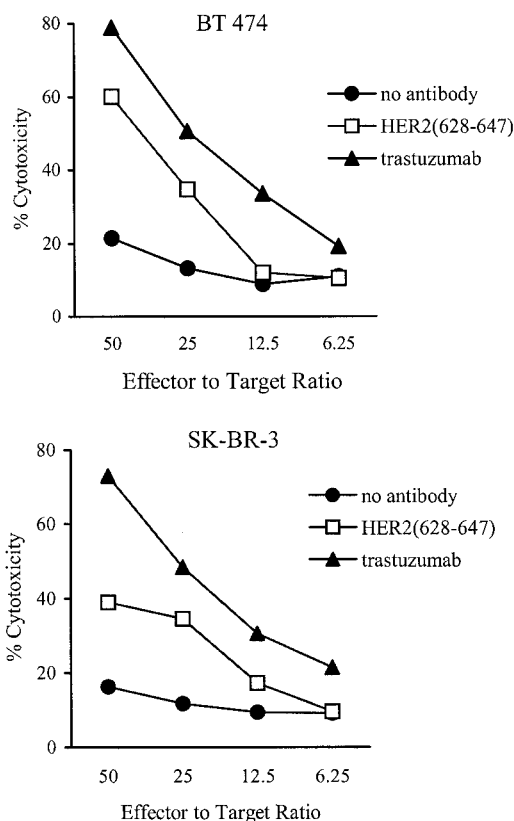


Fig. 7. HER-2(628–647) peptide antibody-mediated ADCC of ^{51}Cr -labeled mammary tumor target cell lines BT-474 (*top*) and SK-BR-3 (*bottom*) was assayed in presence of human PBMCs. The percentage of cytotoxicity was calculated as described in “Materials and Methods.” SDs were less than 10% of the maximum individual values. Less than 2% lysis was observed when target cells were incubated with normal mouse immunoglobulin or antibodies alone without effector cells.

The antitumor activity of HER-2 monoclonal antibodies, some of which recognize denatured protein (22, 52, 53), prompted us to test the efficacy of antibodies raised against peptide immunogens from HER-2. In contrast to peptide vaccine approaches that have focused on multiple T-cell epitopes derived from HER-2 and the generation of anti-HER-2 T-cell responses (34), we have focused on individual B-cell determinants and optimizing an antibody response that has the potential of interfering with the transforming activity of HER-2. We demonstrate that a conformationally optimized chimeric B-cell peptide immunogen that incorporates a promiscuous T-helper epitope elicits high-titered antibodies to HER-2 in both outbred rabbits and inbred transgenic mice. Antibodies elicited by the chimeric peptide MVF HER-2(628–647) had antitumor activity *in vitro* and prevented tumor development *in vivo*. MVF HER-2(628–647) targets a membrane proximal region of the HER-2 ECD, which may be important in the antitumor activity observed with this immunogen. The epitope recognized by the clinically applied HER-2 monoclonal antibody Trastuzumab is reported to be located in the membrane proximal region of the ECD, amino acids 529–627 (54). This antibody has been shown to induce HER-2 receptor degradation (13), inhibit receptor cross-talk (55), and mediate ADCC (27, 54). It is possible that the peptide antibodies elicited by HER-2(628–647) may inhibit tumor growth by these mechanisms as represented by their capacity to mediate ADCC. Optimal cysteine disulfide pairings in the sequence 628–647 have been shown to be critical for transforming activity of HER-2/neu. Disruption of disulfide bonds in this region has been shown to promote ligand-independent receptor homodimerization and early tumor development (56, 57).

Although HER-2(376–395) MVF peptide elicited native receptor-specific antibodies and inhibited tumor cell proliferation in tissue culture, we were unable to evaluate the immunoprotective capacity of this construct in neu transgenic mice because these peptide antibodies failed to cross-react with rat neu receptor. Not all antibodies elicited demonstrated antiproliferative activity. An increase in tumor proliferation was observed *in vitro* with the antibodies elicited by HER-2(410–429) MVF. Monoclonal antibodies to different HER-2 epitopes have demonstrated differential effects. Some bind and display no activity, whereas others stimulate or inhibit tumor growth (22, 52, 53, 58). The predicted B-cell epitopes were not equally immunogenic nor did they equally elicit antibody that bound native HER-2 protein. HER-2(115–136) MVF and HER-2(410–429) MVF were poorly immunogenic in transgenic mice. Strong immune responses to HER-2(376–395) prompted us to evaluate the adjacent epitope HER-2(410–429), although it had the lowest predicted score of the final 12 epitope selections. This low score may explain its poor immunogenicity. Glycosylation is shown to play a decisive role in the immunogenicity of tumor-associated antigens (59) with effects on both the structure of the B-cell determinants and their ability to bind antibodies (60, 61). The inability of HER-2(115–136) MVF peptide antibodies to recognize the receptor might be due to the absence of sugars at the predicted *N*-linked glycosylation site (NNTT) in the synthetic peptide in contrast to other immunogens. This observation is supported by the fact that the antibodies raised against HER-2(115–136) MVF peptide were able to immunoprecipitate the rat neu receptor (Fig. 4). It is interesting to note that rat neu receptor does not harbor a *N*-linked glycosylation site in the analogous region (2).

It is not clear why only a minority of patients with tumors that overexpress HER-2 have manifested objective clinical responses with passive immunotherapy with Trastuzumab (19, 21). Tissue distribution and levels and the inherent immunogenicity of monoclonal antibodies, even humanized constructs, are a few of the major constraints associated with passive therapy. It should also be noted that antitumor activity of HER-2 antibodies in preclinical studies has been only partial and is cytostatic in nature. Because of this, prolonged therapy is necessary. The cost and antigenicity of monoclonal antibody also pose

major obstacles for this application. In contrast, synthetic subunit peptide vaccines are attractive when targeting proteins such as HER-2. Oligopeptide vaccines are cost-effective to produce and are more readily characterized during their manufacture. More importantly, subunit peptide vaccines can focus immune responses to biologically active epitopes. The capacity to narrowly focus the immune response is of particular relevance to HER-2, where interaction of the antibody with specific sites has the potential of stimulating growth. In contrast to passive therapy, the continuous availability of tumor-targeting antibodies can be ensured at low cost. Here we report a B-cell epitope vaccine capable of eliciting HER-2-specific antibodies in an outbred population with a potential to suppress the development of HER-2-overexpressing cancers. A National Cancer Institute-supported Phase I clinical trial to evaluate both the immunogenicity and toxicity of MVF HER-2(628–647) is currently under way at the Ohio State University Medical Center.

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