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Advances in Brief

Existent T-Cell and Antibody Immunity to HER-2/neu Protein in Patients with Breast Cancer[1]

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Abstract

The HER-2/*neu* protooncogene is amplified and overexpressed in 20–40% of invasive breast cancers. HER-2/neu protein overexpression is associated with aggressive disease and is an independent predictor of poor prognosis in several subsets of patients. The protein may also be related to cancer formation, with overexpression being detectable in 50–60% of ductal carcinomas *in situ*. It has been suggested that it might be possible to develop specific T-cell therapy directed against proteins involved in malignant transformation. One question is whether normal proteins that are overexpressed are appropriate targets for therapeutic immune attack. This report demonstrates that some patients with HER-2/neu-positive breast cancers have both existent CD4+ helper/inducer T-cell immunity and antibody-mediated immunity to HER-2/neu protein. Initial studies performed on 20 premenopausal breast cancer patients identified antibodies to HER-2/neu in 11 individuals. Similar antibody responses have been found in some normal individuals. The patient with the greatest antibody response was studied in detail. In addition to a humoral immune response this patient had evidence of a significant proliferative T-cell response to the HER-2/neu protein and peptides. Similar T-cell responses have been detected in additional patients. It has been assumed that patients would be immunologically tolerant to HER-2/neu as a self-protein and that immunity might be difficult to generate. If immunity could be generated, the result might be destructive autoimmunity. The current data support the notion that HER-2/neu-specific immunity might be used in therapy without destroying normal tissue but also raises questions as to the role of existent immunity in immune surveillance and cancer progression.

Introduction

The HER-2/*neu* protooncogene encodes a receptor-like transmembrane protein with a relative molecular mass of 185 kD (p185) and homology to epidermal growth factor receptor (1). The function of HER-2/neu protein is not well defined but is associated with increased tyrosine kinase activity (2). Several ligands have been proposed for HER-2/neu (3, 4) which can induce either stimulatory or inhibitory signals, depending upon ligand and/or experimental conditions (3, 5). In the rat, the protooncogene is activated by a transforming point mutation in the transmembrane domain (1). In human breast cancer no point mutations have been found (6), and the transforming activity is thought to be related to overexpression of protein with normal structure. Overexpression of the HER-2/neu protein has been identified in many human cancers, largely adenocarcinomas of breast, ovary, colon, and lung, and is linked with poor prognosis in subsets of patients with breast cancer (7). HER-2/neu protein is selectively overexpressed by malignant cells and thus has been considered as a possible target for T-cell and antibody-mediated immunotherapy (8). HER-2/neu protein is expressed during fetal development. In adults it is detectable in small amounts in a limited number of normal tissues (9). The assumption has been that any immune response might be selective and have minimal toxicity on normal tissues expressing basal levels of HER-2/neu protein. An alternative view is that normal individuals would be tolerant to HER-2/neu as a self-protein and that if tolerance were circumvented by therapy the result might be autoimmunity directed against HER-2/neu-positive normal tissues. As preliminary studies to the development of HER-2/neu specific immunotherapy, the current studies were initiated to evaluate whether base-line immunity to HER-2/neu existed in patients with breast cancer. Several patients were found in initial studies to have antibody responses to HER-2/neu protein. The one patient with the highest antibody response reported herein was studied in detail and was determined to have both existent antibody and T-cell immunity to the overexpressed oncogenic protein. A more extensive evaluation of patients and normals is required to determine the frequency with which immunity occurs and to determine the extent to which immunity correlates with disease parameters.

Materials and Methods

Patient History. The patient is a 63-year-old female who was first diagnosed with breast cancer in 1988. She had a family history of breast cancer, with both her sister and daughter diagnosed with the disease. At the time of mastectomy she was found to have a 4 x 4 x 3.5-cm left breast mass that was 80% high grade infiltrating ductal carcinoma and 20% intraductal carcinoma. Immunocytochemistry revealed an estrogen receptor level of 5 pmol and nondetectable progesterone receptor. The HER-2/neu protein was positive. Three of 10 axillary lymph nodes were positive for metastatic disease. She was treated with 50 Gy to the affected breast and axilla and received adjuvant chemotherapy of cyclophosphamide, 5-fluorouracil, and methotrexate for 6 months after surgery. After the end of that therapy, she was placed on p.o. tamoxifen. There was no evidence of disease until April 1992 when an abnormal mammogram indicated abnormalities in the opposite breast. Biopsy revealed a 1.2 x 0.6 x 0.5-cm infiltrating ductal carcinoma with a 20% component of ductal carcinoma *in situ* with comedo features. Immunocytochemistry showed an estrogen receptor/progesterone receptor-negative-HER-2/neu protein-positive tumor. Due to other medical reasons, the patient underwent biopsy alone followed by local radiation therapy to the area of tumor. Now, 1 year from the development of her most recent cancer, she has no evidence of local or systemic disease.

Human Sera and Lymphocytes. Sera and lymphocytes were obtained with informed consent. The sera were stored at −20°C prior to testing. PBMC were isolated from heparinized peripheral blood by Ficoll/Hypaque density gradient centrifugation. Cells were washed, aliquoted to concentrations of 2×10^7 PBMC,[3] and resuspended in RPMI 1640 (Gibco, Grand Island, NY) with 2.5×10^{-5} M 2-mercaptoethanol, 200 units/ml penicillin, 200 units/ml strep-

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[3] The abbreviations used are: PBMC, peripheral blood mononuclear cells; MHC, major histocompatibility complex; ICD, intracellular domain; SI, stimulation index; ECL, enhanced chemoluminescence.

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tomycin, 10 mM L-glutamine, and 10% fetal calf serum; 10% dimethyl sulfoxide was added. The cells were then cryopreserved. Sera and lymphocytes were also obtained from normal donors after informed consent. They were processed in an identical fashion.

Synthetic Peptides. Three peptides were constructed, derived from the amino acid sequence of the HER-2/neu protein. The peptides, 15 amino acids in length, were chosen based on an increased probability of binding to Class II MHC molecules. This theoretical potential was evaluated by the use of a protein sequence analysis package, *TSites*, that incorporates several computer algorithms designed to distinguish potential sites for T-cell recognition (10). Two searching algorithms were used: (*a*) the AMPHI algorithm described by Margalit (10, 11) identified epitope motifs according to α helical periodicity and amphipathicity; (*b*) the Rothbard and Taylor algorithm identified epitope motifs according to charge and polarity patterns (12). Using this analysis, we identified more than 40 potential T-cell epitopes in the HER-2/neu protein corresponding to the AMPHI and the Rothbard motifs that would have the potential for binding to Class II MHC molecules. From this group, three peptides were chosen for synthesis, p42–56 (HLDMLRHLYQGCQVV), p783–797 (SRLLGICLTSTVQLV), and p1166–1180 (TLERPKTLSPGKNGV). The peptides were synthesized and purified by Dr. P. S. H. Chou (University of Washington, Seattle, WA), then dissolved in phosphate-buffered saline (pH 7.4) to give 2 mg/ml stock solutions. Prior to aliquoting they were sterile filtered, then stored at −70°C.

p185$^{HER\text{-}2/neu}$ Protein. Crudely purified p185$^{HER\text{-}2/neu}$ for T-cell proliferation assays as well as antibody analysis was obtained from two cell lines, either SKBR3, a human breast cancer line, or NIH 3T3 cells that had been transfected with the c-HER-2/neu DNA (13). In both cell lines, HER-2/neu is a transmembrane protein and was purified from the cell membrane fraction by modifications of described methods for other membrane-associated proteins (14). Briefly, 1×10^6 cells were harvested, washed, and resuspended in phosphate-buffered saline with added protease inhibitors (1 mM benzamidine, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). The cells were then sonicated on ice for 30 s. After documenting cellular disruption, the membrane fraction was separated from the cytosolic portion by ultracentrifugation. Presence of p185 in the membrane fraction was documented by Western blot.

Baculovirus Construction and Purification of Intracellular Domain of HER-2/neu Receptor. A recombinant baculovirus containing sequences encoding the ICD of the HER-2/neu receptor was constructed by using standard methods as described previously (15). Briefly, oligonucleotide polymerase chain reaction primers carrying an *Eco*RV restriction endonuclease site were used to amplify a DNA sequence encoding the entire intracellular domain of the HER-2/neu receptor. Following cleavage with *Eco*RV, the amplified DNA was cloned into the baculovirus vector pAc360. The cloning site in pAc360 was the unique *Bam*HI site which had been repaired by using the Klenow polymerase to fill in the 5′ overhangs. Following sequencing, this vector was used to construct a recombinant virus by standard methods. Recombinant HER-2/neu protein was expressed in Sf9 insect cells essentially as described previously (15). The recombinant fusion protein, which contains 11 amino acids of the baculovirus polyhedrin protein fused to the intracellular domain of the HER-2/neu protein, was purified from Sf9 cell lysates by sequential chromatography on DEAE-Sepharose, phenyl-Sepharose, and Mono Q fast protein liquid chromatography.

Western Blot Analysis for Human Antibody Response. Two methods of electrophoresis were used, either standard electrophoresis in a vertical apparatus, or by the Pharmacia Phast System (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). p185$^{HER\text{-}2/neu}$ and ICD proteins were resolved on a 7.5% polyacrylamide gel in either case. After transfer to nitrocellulose (Hybond-C, Amersham) p185$^{HER\text{-}2/neu}$ proteins were identified by immunoblotting in an identical manner. All control blots were developed by using an IgG1 mouse monoclonal primary antibody commercially prepared, c-neu Ab3 (Oncogene Science). The primary antibody was used in a 1:1000 dilution with tris-buffered saline/1% bovine serum albumin and 0.1% Nonidet P-40. A polyclonal antimouse-horseradish peroxidase-conjugated second antibody (Amersham) was used in a 1:10,000 dilution. The blot was then developed by using a chemiluminescent reaction (Amersham ECL). Identically run experimental blots were analyzed with the patient's sera as primary antibody. The sera were used in a 1:1,000 dilution with tris-buffered saline/1% bovine serum albumin and 0.1% Nonidet P-40. Secondary antibody concentrations were as previously described, except that the secondary antibody was a polyclonal antihuman-horseradish peroxidase conjugate (Amersham). The blots were developed in equal parts of ECL detection reagents (Amersham) for 1 min. after which they were exposed to Hyperfilm-ECL (Amersham), the film was developed and examined for reaction to p185$^{HER\text{-}2/neu}$.

Proliferation Assay. The patient's PBMC were thawed and washed; 2×10^5 cells/well were plated into 96-well round bottomed microtiter plates (Corning, Corning, NY), with 24 replicates per experimental group. The media used consisted of equal parts of EHAA 120 (Biofluids) and RPMI 1640 (Gibco) with L-glutamine, penicillin/streptomycin, 2-mercaptoethanol, and 10% AB serum (human AB CELLect; ICN Flow, Costa Mesa, CA). Experimental groups consisted of 24 wells incubated with PBMC and no antigen, tetanus toxoid (5 μg/ml), p185$^{HER\text{-}2/neu}$ at an estimated 5 μg/ml, and the three derived peptides, individually, at 50 μg/ml. After 4 days, wells were pulsed with 1 μCi of [^{3}H]thymidine for 6–8 h and then counted.

Results

Antibodies to HER-2/neu Are Present in Patient Sera. Initial studies evaluated antibody responses to p185$^{HER\text{-}2/neu}$ protein partially purified from a membrane preparation of SKBR3. SKBR3 is a commonly used human breast cancer cell line with increased HER-2/*neu* gene copy number and overexpression of the p185$^{HER\text{-}2/neu}$ protein (16). Immunoblot analysis, using a commercially prepared antibody to HER-2/neu protein, showed a strong band at 185 kD in evaluation of membrane preparations of SKBR3. Using the same membrane preparations, similarly prepared blots were analyzed by using the patient's sera as primary antibody. Preliminary screening studies (data not shown) demonstrated the presence of antibodies directed toward p185 derived from the SKBR3 membrane preparation in 11 of 20 selected premenopausal patients tested. The patient with the strongest antibody response to p185 was utilized for the studies to be presented below to validate existent immunity to HER-2/neu protein. Sera and lymphocytes were collected on this patient. Fig. 1 shows the Western blot of the patient serum antibody response against the p185$^{HER\text{-}2/neu}$ protein.

To further verify that the patient antibody responses to the 185 kD band was to p185$^{HER\text{-}2/neu}$ and not to possible contaminant allogeneic human proteins in the same band, the results were confirmed by using a different cell line, murine NIH 3T3, that had been transfected with human HER-2/neu complementary DNA (13). Immunoblots with control anti-HER-2/neu monoclonal antibody confirmed the presence of p185$^{HER\text{-}2/neu}$ protein in the membrane preparation of HER-2/neu transfected NIH 3T3 cells, but not in nontransfected wild-type NIH 3T3 cells (Fig. 2). Analysis of patient sera revealed responses to p185$^{HER\text{-}2/neu}$ in membrane preparations of the transfected cells. No similar responses were detected against membrane preparations from nontransfected cells (Fig. 2).

Antibody Responses Can Be Detected against Epitopes Located in Intracellular Domain of HER-2/neu Protein. To partially delineate the epitopes being recognized by the HER-2/neu specific antibody, an immunoblot analysis was performed by using the patient's sera against recombinant intracellular domain protein. A strong response was detected (Fig. 3).

A Proliferative T-Cell Response Was Detected toward Both Intracellular and Extracellular Domain Peptides. In general, immunoglobulin class switching occurs in the presence of cogent T-cell help. The detection of IgG responses to HER-2/neu protein provided inferential evidence for the possible coexistence of helper T-cells recognizing cognate epitopes on the same protein. Helper T-cell responses to p185$^{HER\text{-}2/neu}$ protein and to selected potential immunodominant epitopes were evaluated with standard proliferation assays. Potential immunodominant epitopes for CD4$^+$ helper T-cell responses were chosen by use of computer algorithms, as described in "Materials and Methods." Peripheral blood lymphocytes from the patient were tested in a proliferation assay for stimulation with partially purified p185$^{HER\text{-}2/neu}$ protein, the HER-2/neu peptides p42–56,

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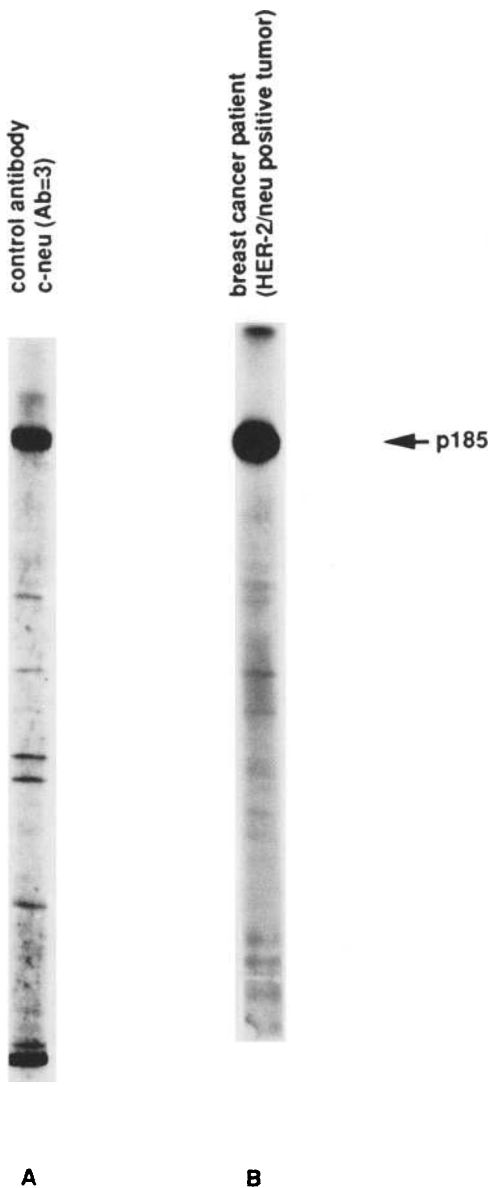

Fig. 1. A patient with a primary breast cancer that overexpressed HER-2/neu protein has antibodies against p185$^{\text{HER-2/neu}}$. The immunoblot in *Lane B* represents the reaction of the patient's sera (1:1000 dilution) against p185$^{\text{HER-2/neu}}$ purified from SKBR3 breast cancer cell line. The control strip in *Lane A* depicts p185$^{\text{HER-2/neu}}$ developed with c-neu Ab3 antibody (Oncogene Science) as the primary antibody (1:1000 dilution). The patient has an antibody response to the HER-2/neu protein.

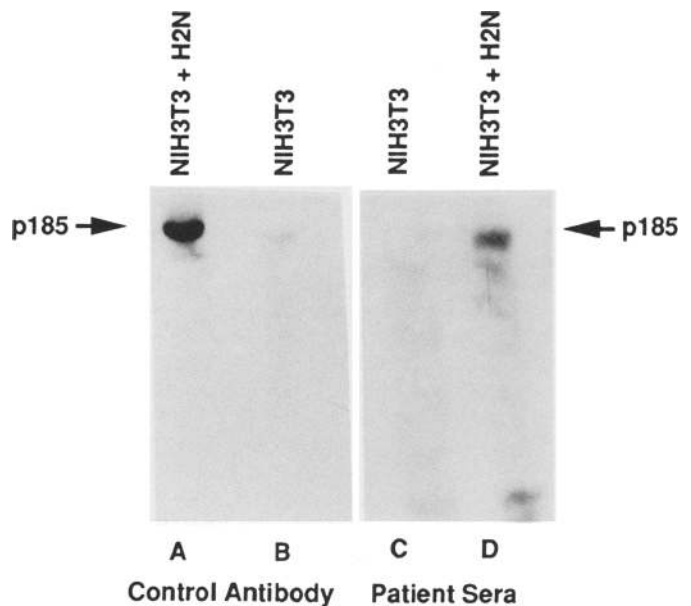

Fig. 2. Serum antibody response to p185$^{\text{HER-2/neu}}$ protein present in membrane preparations of murine NIH 3T3 cells transfected with HER-2/neu complementary DNA. *Lanes A* and *B* show immunoblots of control murine anti-HER-2/neu antibody against transfected and nontransfected NIH 3T3 cells, validating that only the transfected cells contain human p185$^{\text{HER-2/neu}}$ protein. *Lanes C* and *D* show the patient's serum antibody response to p185$^{\text{HER-2/neu}}$ protein in the transfected cell membrane preparation, but no response to the wild-type cells.

p783–797, and p1166–1180, and to tetanus toxoid as a positive control for primed recall responses. Results analyzed as a standard tritiated-thymidine uptake SI demonstrated a substantial response to p185$^{\text{HER-2/neu}}$ protein (SI = 4), p42–56 (SI = 4), and p783–797 (SI = 5), but no response to p1166–1180 (SI <2). (Fig. 4). The detection of proliferation in response to HER-2/neu protein and peptides implies the existence of a primed response as unprimed responses are considered to be undetectable in the same assays.

Seven normal volunteer sera were analyzed in identical assays for antibody and T-cell responses. One individual had antibody response to intracellular domain recombinant protein and another had an antibody response to p185$^{\text{HER-2/neu}}$ in the NIH 3T3 membrane preparation. Peripheral blood from the 7 normal donors was analyzed in a proliferation assay in a duplicate fashion. Primed tetanus toxoid responses were validated, but no responses to HER-2/neu protein or peptides were detected. An extensive evaluation of breast cancer patients and normal donors has not yet been performed to determine the true frequency with which immunity occurs or to determine the extent to which immunity correlates with disease parameters.

Discussion

Our evaluation of the immune response to p185$^{\text{HER-2/neu}}$ oncoprotein revealed several patients with existent immunity antibody responses, one of which was studied in detail here. The HER-2/*neu* antibodies detected were IgG, implying that cognate helper T-cell immunity might be present and operative. T-cell immunity was evaluated in the patient with the strongest antibody response. T-Cell responses in cancer patients to overexpressed oncogenic proteins have not been previously described. Human proliferative T-cell responses have been detected against mutated *ras* peptides (17), but these responses were elicited to mutated segments of protein uniquely present in cancer cells (*i.e.*, putative cancer-specific antigens). The patient evaluated in the current report had a marked proliferative helper T-cell response to the protein and two immunodominant peptide epitopes were defined. The level of proliferative response detected was consistent with a primed response, raising the possibility that her tumor was able to act as a source of antigen with determinants capable of interacting with class II MHC and within the host T-cell receptor repertoire. CD4$^+$ helper/inducer T-cell responses are normally directed to soluble antigen presented by antigen-presenting cells. It is known that the extracellular domain portion of the protein is soluble and present in the extratumor environment, therefore available for stimulation of immune T-cells (18). Existent responses were found to epitopes derived from both the extracellular (p42–56) and intracellular (p783–797) portion of the protein, indicating both segments are available to the Class II MHC antigen-processing pathway.

Antibody was detected to p185 and to the ICD. Immunity to the extracellular domain is currently being evaluated. There have been reports of antibody responses to other protooncogene-encoded proteins in cancer patients, including c-myc, c-myb, and p53 (19–21). These other oncogene proteins are normally intracellular. Antibody to intracellular proteins would not be expected to affect normal cell function. Immunogenicity could be explained by exposure of previ-

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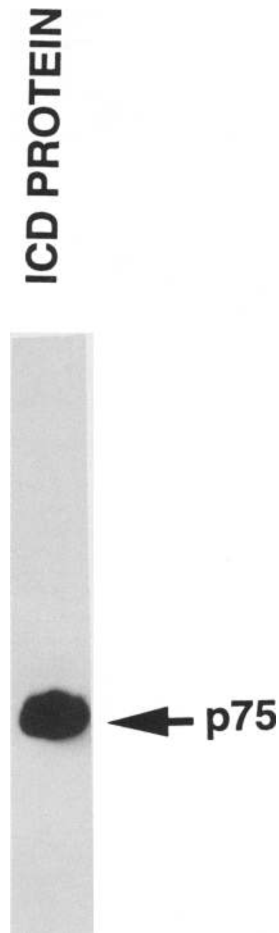

Fig. 3. Antibody responses can be detected against epitopes located in the intracellular domains. Sera from the patient described were used as primary antibody in an immunoblot using recombinant intracellular domain protein (described in "Materials and Methods"). The patient had a marked response to the intracellular domain protein.

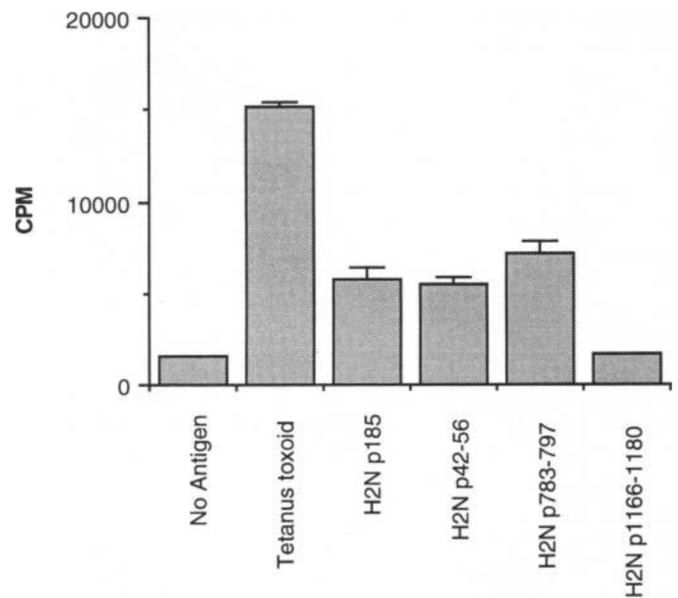

Fig. 4. Primed helper T-cells response to $p185^{HER\text{-}2/neu}$ protein and to peptide epitopes located in both the extracellular and intracellular domains. Peripheral blood lymphocytes from the patient were evaluated for T-cell proliferation in response to stimulation by HER-2/neu protein partially purified from SKBR3 membrane and in response to synthetic peptides derived from both the ECD and ICD. The data represents the mean of 24 determinations of the c.p.m. with standard error bars expressed. Primed T cell response were detected against HER-2/neu protein and against peptides p42–56 and p783–797 from the extracellular domain and ICD, respectively, as well as to control tetanus toxoid.

ously sequestered proteins to the immune system by mechanisms related to malignancy such as tumor necrosis. HER-2/neu is a transmembrane growth factor receptor. Antibody to the extracellular domain might profoundly alter cell function by stimulating the receptor or by blocking the function of the normal ligand(s). As examples, heterologous antibodies against HER-2/neu have been shown to provide either agonistic or antagonistic effects dependent upon the particular antibody (22). Natural ligands for HER-2/neu have been reported to have variable effects depending upon the concentration (4, 5). Since helper T-cell immunity was detected and since antibody tolerance to self-proteins in some circumstances is due to helper T-cell tolerance and not deletion of self-reactive B-cells, it is possible that the existent antibody response is in part directed against the ECD.

Of critical importance is the role of detected T-cell immunity in tumor progression. In animal models, immune T-cells often coexist with progressive fatal tumors, and the animal will die of disease despite existent immunity. If immunity is eradicated, however, the time to death is more rapid. In such models, the immune T-cells that are ineffective, or only partially effective in the primary host can be made effective in therapy by *in vitro* activation and growth, and treating the animal with increased numbers of the cultured T-cells. Thus, HER-2/neu immunity might be augmented to provide therapeutic benefit. Alternatively, immunity to HER-2/neu protein could possibly lead to tumor stimulation by release of inappropriate cytokines at sites of tumor deposition. Whether anti-HER-2/neu responses are beneficial or detrimental in patients is important to define before augmenting that immunity in an effort to eradicate malignant cells.

Tumor immunosurveillence is a much debated concept, in part, because few antigen systems are available to confirm or deny any hypothesis. The presence of immunity to the HER-2/neu protein in patients with breast cancer now provides that system. Although HER-2/neu protein overexpression is associated with 20–40% of frank breast carcinomas, it is found in 50–60% of ductal carcinoma *in situ* (23). The decrease in HER-2/neu-positive cells in invasive ductal carcinomas may possibly be due to immune surveillance. If that hypothesis is correct, then T-cells in some patients who have HER-2/neu positive ductal carcinoma *in situ* may be able to totally eradicate malignant cells and prevent the development of invasive carcinomas, whereas T-cell immunity in other circumstances might select for HER-2/neu-negative cancer cells. HER-2/neu-positive invasive cancers that are able to progress in the presence of immunity would presumably be biologically the most aggressive.

The current report focused on the elucidation of the immune response in a single patient with a high antibody response. We have detected antibody responses to the p185HER-2/neu protein in other patients, as well as in some normal individuals, but they have not yet been studied in detail. Correlation of response in large numbers of patients with protein overexpression, stage, and histology of the primary breast cancer, and clinical outcome must be determined. Large numbers of age-matched nonaffected individuals need to be studied as well. If the concept of immunosurveillance is valid, the detection of immune responses might be used as a tool for early diagnosis or screening. This would be the case if antibody response level or epitopes recognized prove to be preferential for patients with malignancy and develop early in the course of the disease, before the tumor is otherwise manifest or large enough for detection by conventional methods. The detection of antibody in several normal individuals would at this point mitigate against that hypothesis, but delineation will require analysis of a large population of normal individuals to determine the false-positive frequency. Whether or not immunity is predictive of malignancy, levels of existent immunity may change in a predictable fashion in response to changes in clinical circumstances,

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such as the recurrence of a HER-2/neu-positive malignancy and aid in the assessment of clinical status.

Development of successful strategies for immunotherapy of human cancers has been an area of intense investigation of several years. While encouraging results have been reported for nonspecific therapies, specific cures for individual malignancies are not yet uniformly available. This demonstration that a patient with a HER-2/neu-positive breast cancer has an immune response toward the HER-2/neu protein supports the concept that oncogenic proteins might serve as tumor antigens in human malignancy. Furthermore, definition of the immunodominant epitopes will aid in identifying potential determinants for use in T-cell vaccines or T-cell-mediated immunotherapy. Since some patients are not tolerant to this overexpressed self-protein, elicitation of immune responses in nonimmune cancer-bearing individuals should be expected. Detection of immunity without any evidence of autoimmune destruction of normal tissue provides some comfort that therapies centered on augmenting T-cell immunity will not induce toxic autoimmunity in humans. HER-2/neu protein overexpression in breast cancer is linked with a younger population, the premenopausal patient, and more aggressive disease. This is the patient population with the highest morbidity and mortality associated with their breast cancers. The results described here should help stimulate investigation into the use of the HER-2/neu oncoprotein as a target for specific immunotherapy of breast cancer.

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Addendum

The ability of patients to mount an immune response against the HER-2/neu protein is further supported by the finding that tumor-associated cytotoxic T-lymphocyte expanded from a patient with ovarian cancer was found to respond to HER-2/neu peptides (24).

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