

p185^{HER2} Monoclonal Antibody Has Antiproliferative Effects In Vitro and Sensitizes Human Breast Tumor Cells to Tumor Necrosis Factor

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The *HER2/c-erbB-2* gene encodes the epidermal growth factor receptorlike human homolog of the rat *neu* oncogene. Amplification of this gene in primary breast carcinomas has been shown to correlate with poor clinical prognosis for certain cancer patients. We show here that a monoclonal antibody directed against the extracellular domain of p185^{HER2} specifically inhibits the growth of breast tumor-derived cell lines overexpressing the *HER2/c-erbB-2* gene product and prevents *HER2/c-erbB-2*-transformed NIH 3T3 cells from forming colonies in soft agar. Furthermore, resistance to the cytotoxic effect of tumor necrosis factor alpha, which has been shown to be a consequence of *HER2/c-erbB-2* overexpression, is significantly reduced in the presence of this antibody.

HER2/c-erbB-2, the human homolog of the rat proto-oncogene *neu* (4, 34), encodes a 1,255-amino-acid glycoprotein with extensive homology to the human epidermal growth factor (EGF) receptor (4, 21, 33, 34, 42). The *HER2/c-erbB-2* gene product, p185^{HER2}, has all of the structural features and many of the functional properties of subclass I growth factor receptors (reviewed in references 43 and 44), including cell surface location and an intrinsic tyrosine kinase activity. However, the ligand for this putative growth factor receptor has not yet been identified.

Amplification of the *HER2/c-erbB-2* gene has been found in human salivary gland and gastric tumor-derived cell lines (13, 34), as well as in mammary gland carcinomas (21, 22, 40, 42). Slamon et al. (35) surveyed 189 primary breast adenocarcinomas and determined that the *HER2/c-erbB-2* gene was amplified in about 30% of the cases. Most importantly, *HER2/c-erbB-2* amplification was correlated with a negative prognosis and high probability of relapse. Similar although less frequent amplification of the *HER2/c-erbB-2* gene has been reported for gastric and colon adenocarcinomas (45, 46). Experiments with NIH 3T3 cells also suggest a direct role for the overexpressed, structurally unaltered *HER2/c-erbB-2* gene product p185^{HER2} in neoplastic transformation. High levels of *HER2/c-erbB-2* gene expression attained by coamplification of the introduced gene with dihydrofolate reductase by methotrexate selection (18) or by using a strong promoter (6) was shown to transform NIH 3T3 fibroblasts. Only cells with high levels of p185^{HER2} are transformed, i.e., have an altered morphology, are anchorage independent, and will form tumors in athymic mice.

Overexpression of p185^{HER2} may, furthermore, contribute to malignant tumor development by allowing tumor cells to evade one component of the antitumor defenses of the body, the activated macrophage (17). Macrophages play an important role in immune surveillance against neoplastic growth in vivo (1, 2, 38), and Urban et al. (39) have shown that tumor

cells made resistant to macrophages display enhanced tumorigenicity. Tumor necrosis factor alpha (TNF- α) has been shown to play a role in activated macrophage-mediated tumor cell killing in vitro (3, 11, 23, 29, 39). NIH 3T3 cells transformed by a transfected and amplified *HER2/c-erbB-2* cDNA show increased resistance to the cytotoxic effects of activated macrophages or TNF- α in direct correlation with increased levels of p185^{HER2} expression. Furthermore, breast tumor cell lines with high levels of p185^{HER2} exhibit resistance to TNF- α . Resistance to host antitumor defenses could facilitate the escape of cells from a primary tumor to establish metastases at distant sites.

To further investigate the consequences of alteration in *HER2/c-erbB-2* gene expression in mammary gland neoplasia and to facilitate investigation of the normal biological role of the *HER2/c-erbB-2* gene product, we have prepared monoclonal antibodies against the extracellular domain of p185^{HER2}. One monoclonal antibody (4D5) was characterized in more detail and was shown to inhibit in vitro proliferation of human breast tumor cells overexpressing p185^{HER2} and, furthermore, to increase the sensitivity of these cells to the cytotoxic effects of TNF- α .

MATERIALS AND METHODS

Cells and cell culture. Human tumor cell lines were obtained from the American Type Culture Collection. The mouse fibroblast line NIH 3T3/HER2-3₄₀₀, expressing an amplified *HER2/c-erbB-2* cDNA under simian virus 40 early promoter control, and the vector-transfected control cell line NIH 3T3/CVN have been described previously (18).

Cells were cultured in a 1:1 mixture of Dulbecco modified Eagle medium and Ham nutrient mixture F-12 supplemented with 2 mM glutamine, 100 u of penicillin per ml, 100 μ g of streptomycin per ml, and 10% serum. Human tumor cell lines were cultured with fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.); NIH 3T3 derivatives were cultured with calf serum (Hyclone Laboratories, Inc., Logan, Utah.).

Immunization. Female BALB/c mice were immunized with NIH 3T3/HER2-3₄₀₀ cells expressing high levels of

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p185^{HER2}. The cells were washed once with phosphate-buffered saline (PBS) and detached from the plate with PBS containing 25 mM EDTA. After low-speed centrifugation, the cells were suspended in cold PBS (2×10^7 cells per ml). Each mouse was injected intraperitoneally with 0.5 ml of this cell suspension on weeks 0, 2, 5, and 7.

On weeks 9 and 13, 100 μ l of a Triton X-100 membrane preparation of p185^{HER2}, partially purified by wheat germ agglutinin chromatography (700 μ g of protein per ml) (25), was administered intraperitoneally. Three days before fusion, 100 μ l of the enriched p185^{HER2} protein was administered intravenously.

Fusion and screening. Mice with high antibody titers as determined by immunoprecipitation of p185^{HER2} were sacrificed, and their splenocytes were fused as described previously (26). Spleen cells were mixed at a 4:1 ratio with the fusion partner, mouse myeloma cell line X63-Ag8.653 (20), in the presence of 50% polyethylene glycol 4000. Fused cells were plated at a density of 2×10^5 cells per well in 96-well microdilution plates. The hypoxanthine-azaserine (12) selection for hybridomas was begun 24 h later. Beginning at day 10 postfusion, supernatants from hybridoma-containing wells were tested for the presence of antibodies specific for p185^{HER2} by an enzyme-linked immunosorbent assay with the wheat germ agglutinin chromatography-purified p185^{HER2} preparation (28). Enzyme-linked immunosorbent assay-positive supernatants were confirmed by immunoprecipitation and cloned twice by limiting dilution.

Large quantities of specific monoclonal antibodies were produced by preparation of ascites fluid; antibodies were then purified on protein A-Sepharose columns (Fermentech, Inc., Edinburgh, Scotland) and stored sterile in PBS at 4°C.

Immunoprecipitations and antibodies. Cells were harvested by trypsinization, counted in a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.), and plated 24 h before being harvested for analysis of p185^{HER2} expression. Cells were lysed at 4°C with 0.8 ml of HNEG lysis buffer (18) per 100-mm plate. After 10 min, 1.6 ml of lysis dilution buffer (HNEG buffer with 1% bovine serum albumin and 0.1% Triton X-100) was added to each plate, and the extracts were clarified by centrifugation at $12,000 \times g$ for 5 min.

Antibodies were added to the cell extracts and allowed to bind at 4°C for 2 to 4 h. Immune complexes were collected by adsorption to protein A-Sepharose beads for 20 min and washed three times with 1 ml of HNEG buffer-0.1% Triton X-100. Autophosphorylation reactions were carried out for 20 min at 4°C in 50 μ l of HNEG wash buffer containing 5 mM MnCl₂ and 3 μ Ci of [γ -³²P]ATP (5,000 Ci/mmol, Amersham Corp., Arlington Heights, Ill.). The autophosphorylation reaction conditions have been described previously (18). Proteins were separated on sodium dodecyl sulfate (SDS)-7.5% polyacrylamide gels and analyzed by autoradiography.

The polyclonal antibody, G-H2CT17, recognizing the carboxy-terminal 17 amino acids of p185^{HER2}, has been described previously (18). The anti-EGF receptor monoclonal antibody 108 (16) was provided by Joseph Schlessinger, Rorer Biotechnology, Inc.

Fluorescence-activated cell sorting. SK-BR-3 human breast tumor cells overexpressing the *HER2/c-erbB-2* gene (17, 22) or A431 human squamous carcinoma cells overexpressing the EGF receptor gene (14) were grown in T175 flasks. They were detached from the flasks by treatment with 25 mM EDTA-0.15 M NaCl, collected by low-speed centrifugation, and suspended at 1×10^6 cells per ml in PBS-1% fetal bovine serum. One milliliter of each cell line was incubated with 10 μ g of either anti-*HER2/c-erbB-2* monoclonal antibody (4D5)

or a control antibody (40.1.H1) recognizing the hepatitis B surface antigen. The cells were washed twice and suspended on ice for 30 min in 1 ml of PBS-1% fetal bovine serum containing 10 μ g of goat anti-mouse immunoglobulin G F(ab')₂ fragments conjugated with fluorescein isothiocyanate dye (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Unbound fluorescein dye was removed by two further washes. The cells were suspended at 2×10^6 per ml in PBS-1% fetal bovine serum and analyzed with an EPICS 753 (Coulter) fluorescence-activated cell sorter. Fluorescein was excited by 300 mW of 488-nm argon laser light, and the emitted light was collected with a 525-nm band-pass filter with a 10-nm band width.

Down-regulation assay. SK-BR-3 cells were plated at 1.5×10^5 cells per 35-mm culture dish in normal medium. After a 6-h period to allow attachment, the medium was replaced by 1.5 ml of methionine-free labeling medium containing 150 μ Ci of [³⁵S]methionine per ml and 2% dialyzed fetal bovine serum. The cells were metabolically labeled for 14 h and then chased with medium containing 2% dialyzed serum and unlabeled methionine. Either a control monoclonal antibody (40.1.H1) or anti-p185^{HER2} (4D5) was added to a final concentration of 2.5 μ g/ml. At 0, 5, and 11 h, extracts were prepared with 0.3 ml of lysis solution and 0.6 ml of dilution buffer. The p185^{HER2} was immunoprecipitated with 2.5 μ l of polyclonal antibody G-H2CT17. The washed immune complexes were dissolved in sample buffer, electrophoresed on a SDS-7.5% polyacrylamide gel, and analyzed by autoradiography. Each time point determination was performed in duplicate. Autoradiograph band intensities were quantitated by using a scanner (Ambis Systems).

Cell proliferation assays. The anti-p185^{HER2} monoclonal antibodies were characterized by using the breast tumor cell line SK-BR-3. Cells were detached by using 0.25% (vol/vol) trypsin and suspended in complete medium at a density of 4

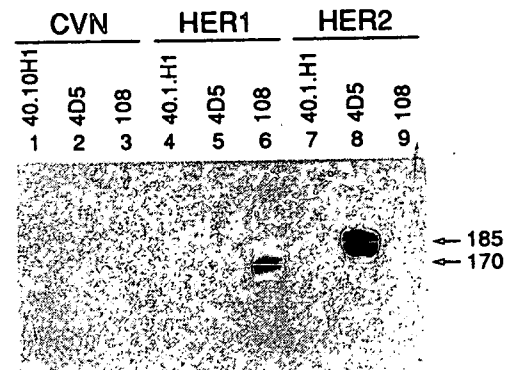


FIG. 1. Specificity of monoclonal antibody 4D5. Three cell lines, NIH 3T3/CVN, NIH 3T3/HER1-EGF receptor, and NIH 3T3/HER2-3₄₀₀, were plated out at 2.0×10^6 in 100-mm culture dishes. At 24 h, Triton X-100 lysates were prepared and divided into three portions. Either an irrelevant monoclonal antibody (6 μ g of anti-hepatitis B virus surface antigen, 40.1.H1; lanes 1, 4, and 7), anti-p185^{HER} monoclonal antibody 4D5 (6 μ g; lanes 2, 5, and 8), or anti-EGF receptor monoclonal antibody 108 (6 μ g; lanes 3, 6, and 9) was added and allowed to bind at 4°C for 4 h. The immune complexes were collected with 30 μ l of protein A-Sepharose. Rabbit anti-mouse immunoglobulin (7 μ g) was added to each 4D5 immunoprecipitation to improve the binding of this monoclonal antibody to the protein A-coated beads. Proteins were labeled by autophosphorylation and separated on an SDS-7.5% polyacrylamide gel. The gel was exposed to film at -70°C for 4 h with an intensifying screen. The arrows show the positions of proteins of *M*_r 185,000 and 170,000.

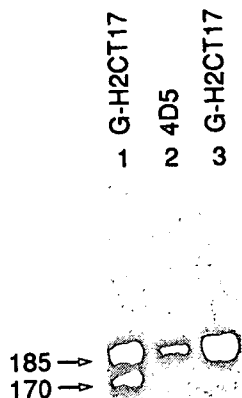


FIG. 2. Binding of monoclonal antibody 4D5 to unglycosylated receptor. NIH 3T3/HER2-3₄₀₀ cells were plated into two 100-mm plates at 2×10^6 cells per plate. After 14 h, the antibiotic tunicamycin was added to one plate at $3 \mu\text{g/ml}$. After a further 5.5 h of incubation, Triton X-100 lysates were then prepared from each plate. Immunoprecipitations, the autophosphorylation reaction, and SDS-polyacrylamide gel electrophoresis were performed as described in the legend to Fig. 1. Lanes: 1, tunicamycin-treated cell lysate (one-third of a plate) immunoprecipitated with $2.5 \mu\text{l}$ of a polyclonal antibody directed against the C terminus of p185^{HER2}; 2, tunicamycin-treated cell lysate (one-third of a plate) immunoprecipitated with $6 \mu\text{g}$ of 4D5; 3, untreated control lysate (one-third of a plate) immunoprecipitated with the polyclonal antibody. The arrows show the locations of proteins of M_r 185,000 and 170,000.

$\times 10^5$ cells per ml. Aliquots of $100 \mu\text{l}$ (4×10^4 cells) were plated into 96-well microdilution plates, the cells were allowed to adhere, and $100 \mu\text{l}$ of media alone or media containing monoclonal antibody (final concentration, $5 \mu\text{g/ml}$) was then added. After 72 h, plates were washed twice with PBS (pH 7.5), stained with crystal violet (0.5% in methanol), and analyzed for relative cell proliferation as described previously (36).

For assays in which monoclonal antibodies were combined with recombinant human TNF- α (5.0×10^7 U/mg; Genentech, Inc.), cells were plated and allowed to adhere as described above. Following cell adherence, control medium alone or medium containing monoclonal antibodies was added to a final concentration of $5 \mu\text{g/ml}$. Cultures were incubated for another 4 h, and then increasing concentrations of TNF- α were added to a final volume of $200 \mu\text{l}$. Following 72 h of incubation, the relative cell number was determined by crystal violet staining. Some samples were analyzed by crystal violet staining following cell adherence for determination of the initial cell number.

RESULTS

Specificity of monoclonal antibody 4D5. Monoclonal antibodies directed against the extracellular domain of p185^{HER2} were prepared by immunizing mice with NIH 3T3 cells transfected with a *HER2/c-erbB-2* cDNA (HER2-3₄₀₀) (17, 18) and overexpressing the corresponding gene product, p185^{HER2}. One antibody exhibited several interesting biological properties and was chosen for further characterization. Antibody 4D5 specifically immunoprecipitated a single ³²P-

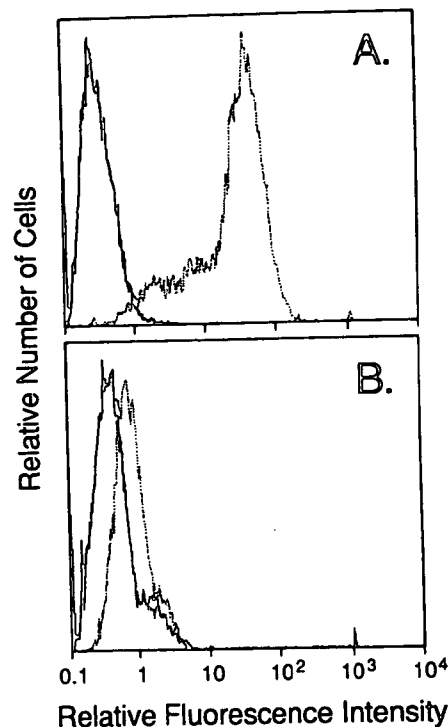


FIG. 3. Fluorescence-activated cell sorter histograms of human tumor cells binding anti-p185 monoclonal antibody 4D5. —, Binding by the control antibody, 40.1.H1, directed against the hepatitis B surface antigen; ·····, binding by the anti-*HER2/c-erbB-2* antibody, 4D5. The antibodies were first allowed to react with the cell surface. After a wash step, bound antibody was labeled by addition of fluorescein-conjugated F(ab')₂ fragment of goat anti-mouse immunoglobulin G. (A) Binding of the antibodies to the human breast tumor line SK-BR-3, which contains an amplification of the *HER2/c-erbB-2* gene and expresses high levels of the *HER2/c-erbB-2* gene product p185^{HER2}. (B) Binding of the same antibodies to the human squamous epithelial cell line A431. This cell line expresses low levels of mRNA for *HER2/c-erbB-2* and high levels (2×10^6 receptors per cell) of the EGF receptor.

labeled protein of M_r 185,000 from NIH 3T3 cells expressing p185^{HER2} (Fig. 1, lane 8). This antibody did not cross-react with the human EGF receptor (HER1; Fig. 1, lane 5), even when overexpressed in a mouse NIH 3T3 background (Fig. 1, lane 6). Furthermore, it did not immunoprecipitate any proteins from NIH 3T3 cells transfected with a control plasmid (pCVN) which expresses the neomycin resistance and dihydrofolate reductase genes only (Fig. 1, lane 2).

To determine the nature of the epitope recognized by 4D5, NIH 3T3/HER2-3₄₀₀ cells were treated with tunicamycin, which prevents addition of N-linked oligosaccharides to proteins (15, 41). Cells treated with this antibiotic for 5.5 h contained two proteins which were immunoprecipitated by a polyclonal antibody against the carboxy-terminal peptide of p185^{HER2} (Fig. 2, lane 1). The polypeptide of 170,000 M_r represents unglycosylated p185^{HER2}. The upper band of ca. 185,000 M_r comigrated with glycosylated p185^{HER2} from untreated cells (Fig. 2, lane 3). Monoclonal antibody 4D5 efficiently immunoprecipitated only the glycosylated form of p185^{HER2} (Fig. 2, lane 2). This experiment suggests either that the epitope recognized by 4D5 consists partly of carbohydrate, or, alternatively, that the antibody recognizes a conformation of the protein achieved only when it is glycosylated.

TABLE 1. Inhibition of SK-BR-3 proliferation by anti-p185^{HER2} monoclonal antibodies^a

Monoclonal antibody	Relative cell proliferation ^b
7C2	79.3 ± 2.2
2C4	79.5 ± 4.4
7D3	83.8 ± 5.9
4D5	44.2 ± 4.4
3E8	66.2 ± 2.4
6E9	98.9 ± 3.6
7F3	62.1 ± 1.4
3H4	66.5 ± 3.9
2H11	92.9 ± 4.8
40.1.H1	105.8 ± 3.8
4F4	94.7 ± 2.8

^a SK-BR-3 breast tumor cells were plated as described in Materials and Methods. Following adherence, medium containing 5 µg of either anti-p185^{HER2} or control monoclonal antibodies (40.1.H1 and 4F4) per ml were added.

^b Relative cell proliferation was determined by crystal violet staining of the monolayers after 72 h. Values are expressed as a percentage of results with untreated control cultures (100%).

The binding of monoclonal antibody 4D5 to human tumor cell lines was investigated by fluorescence-activated cell sorting (Fig. 3). This antibody was bound to the surface of cells expressing p185^{HER2}. Figure 3A shows the 160-fold increase in cellular fluorescence observed when 4D5 was added to SK-BR-3 breast adenocarcinoma cells relative to a control monoclonal antibody. This cell line contains an amplified *HER2/c-erbB-2* gene and expresses high levels of p185^{HER2} (17, 22). In contrast, the squamous carcinoma cell line A431, which expresses about 2×10^6 EGF receptors per cell (14) but only low levels of p185^{HER2} (4), exhibited only a twofold increase in fluorescence with 4D5 (Fig. 3B) when compared with a control monoclonal antibody.

The binding of 4D5 correlated with the levels of p185^{HER2} expressed by these two cell lines. SK-BR-3 cells, expressing high levels of p185^{HER2}, showed an 80-fold increase in relative fluorescence intensity compared with A431 cells. This experiment demonstrates that 4D5 specifically recognizes the extracellular domain of p185^{HER2}.

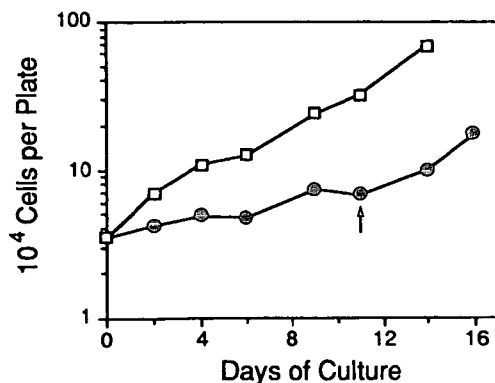


FIG. 4. Growth curve of SK-BR-3 cells treated with anti-*HER2/c-erbB-2* monoclonal antibody 4D5. Cells were plated into 35-mm culture dishes at 20,000 cells per plate in medium containing 2.5 µg of either control antibody (40.1.H1, anti-hepatitis B surface antigen) (□) or anti-p185^{HER2} antibody 4D5 (●) per ml. On the indicated days, cells were trypsinized and counted in a Coulter counter. The determination for each time point and each antibody was done in duplicate, and the counts were averaged. The arrow indicates the day the cells were refed with medium without antibodies.

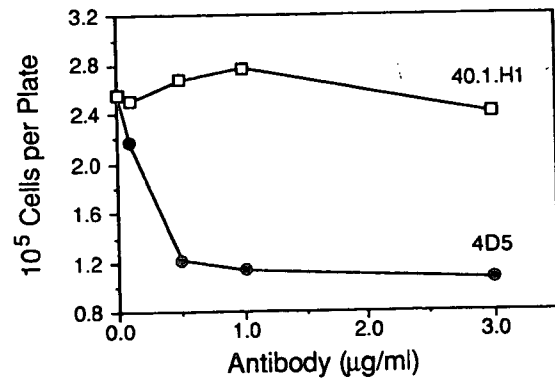


FIG. 5. Growth of SK-BR-3 cells in different concentrations of monoclonal antibody 4D5. The human breast tumor line SK-BR-3 was plated into 35-mm culture dishes at 20,000 cells per dish. Either 0.1, 0.5, 1.0, or 3.0 µg of a control monoclonal antibody (40.1.H1, anti-hepatitis B surface antigen) or monoclonal 4D5 antibody per ml was added at the time of plating. After 8 days of growth, the plates were trypsinized and the cells were counted in a Coulter counter. Each concentration of antibody was plated and counted in duplicate, and the cell numbers were averaged.

Effects on cell proliferation. We used the human mammary gland adenocarcinoma cell line, SK-BR-3, to determine whether monoclonal antibodies directed against the extracellular domain of p185^{HER2} had any effect on the proliferation of cell lines overexpressing this receptorlike protein. SK-BR-3 cells were coincubated with several *HER2/c-erbB-2*-specific monoclonal antibodies or with either of two different control monoclonal antibodies (40.1.H1, directed against the hepatitis B surface antigen; 4F4, directed against recombinant human gamma interferon). Most anti-*HER2/c-erbB-2* monoclonal antibodies which recognize the extracellular domain inhibited the growth of SK-BR-3 cells (Table

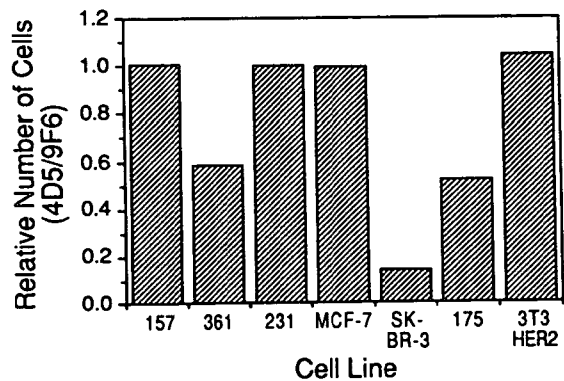


FIG. 6. Screening of breast tumor cell lines for growth inhibition by monoclonal antibody 4D5. Each cell line was plated in 35-mm culture dishes at 20,000 cells per dish. Either a control monoclonal antibody (9F6, anti-human immunodeficiency virus gp120) or the anti-p185^{HER2} monoclonal antibody 4D5 was added on day 0 to 2.5 µg/ml. Because the different cell lines grow at different rates, the cell lines NIH 3T3/*HER2-3₄₀₀* and SK-BR-3 were counted after 6 days, cell lines MDA-MB-157, MDA-MB-231, and MCF-7 were counted after 9 days, and cell lines MDA-MB-175V11 and MDA-MB-361 were counted after 14 days. The difference in growth between cells treated with 4D5 and 40.1.H1 is expressed as the ratio of cell numbers with 4D5 versus a control monoclonal antibody, 9F6. Each cell line was assayed in duplicate for each antibody, and the counts were averaged.

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