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The erbB family of tyrosine kinase receptors includes epidermal growth factor receptor, erbB-2 (Her-2), erbB-3 (Her-3), and erbB-4 (Her-4; Ref. 1). These transmembrane receptors are involved in the regulation of various physiological processes, including cell growth, differentiation, cell-cell interactions, and cytokine signaling (2, 3). The overexpression of the erbB-2 (Her-2) receptor occurs in various human cancers, including breast, ovary, prostate, gastric, lung, bladder, and kidney carcinomas (4) and is generally associated with an enhanced metastatic potential and a poor prognosis (5). The levels of Her-2 expression correlate with the degree of breast tumor differentiation, estrogen receptor status, and clinical outcome (6). Several mechanisms are responsible for the aggressive behavior of Her-2+ tumor cells (7–9). These include the up-regulation of growth factor-mediated tumor angiogenesis that has been recently associated with the overexpression of Her-2 (10–13). One of these proangiogenic factors, VEGF3, is down-regulated by antibodies against Her-2, resulting in the inhibition of tumor growth (14, 15). Because Her-2 is expressed at lower levels on cells in many normal tissues (16), it makes an attractive target for immunotherapy.

There has been considerable interest in developing agents that block the signaling function of Her-2, as well as VEGF secretion or activity. Particularly effective are the MAbs raised against Her-2, which inhibit the in vitro and in vivo growth of Her-2-overexpressing tumor cells (17, 18). Several anti-Her-2 MAbs recognizing different epitopes on the EC domain of Her-2 have been described, and the epitopes recognized have been mapped to different parts of the molecule (19–23). The humanized anti-Her-2 MAb, 4D5 (Herceptin), has been approved by the Food and Drug Administration for human use and has increased the survival of relapsed patients with Her-2-overexpressing breast tumors (24, 25).

In this study, we demonstrate that MAbs against several epitopes on Her-2 make highly effective antitumor agents in vivo when used as a mixture. The MAb mixture is superior to individual MAbs in vitro with regard to inhibiting tumor cell growth and VEGF secretion, as well as mediating ADCC and CDC.

Materials and Methods

Cells. The human breast cancer cell line BT474 (American Type Culture Collection, Manassas, VA) was maintained in monolayer culture in MEM (Life Technologies, Inc., Grand Island, NY) supplemented with 1% vitamins, 2 mM l-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 1% HEPES buffer (all from Life Technologies, Inc.), and 10% heat-inactivated FCS (Hyclone, Logan, UT). Cultures were grown at 37°C and 5% CO2, and cells were passaged when they were 70% confluent. Before passage, cells were removed from the flasks after a 5-min incubation with trypsin/EDTA (Life...
M Abs. Mouse anti-Her-2 M Abs were generated in our laboratory by immunizing BALB/c mice s.c. with the EC domain of Her-2. Spleen cells from the immunized mice were harvested and fused with the myeloma cell line SP2/0. The hybridomas were subcloned and assayed by ELISA for their ability to secrete immunoglobulin. Immunoglobulin-containing SNs from positive clones were tested by ELISA for reactivity against the EC domain of Her-2. Positive SNs were then tested by indirect fluorescence on a panel of breast cancer cell lines and analyzed on a FACS (Becton Dickinson, Franklin Lakes, NJ). The M Abs were affinity purified on protein G-Sepharose (Pharmacia, Piscataway, NJ) and further characterized.

Characterization of M Abs. Twelve M Abs recognizing nine different epitopes on Her-2 were characterized for binding to BT474 cells using indirect immunofluorescence assays and for epitope specificity by cross blocking in direct immunofluorescence assays. Indirect immunofluorescence assays were carried out on BT474 cells suspended at 1 × 10⁶/ml in CM and treated with 0.01–1 µg/ml M Abs for 30 min on ice. The excess M Abs were washed out with CM containing 0.05% sodium azide, and the cells were then treated with 2 µl of the secondary FITC-labeled goat antimouse immunoglobulin and analyzed on a FACScan to determine the percentage of cells that are stained and their MFI.

Direct immunofluorescence assays were carried out with FITC-anti-Her-2 M Abs to determine the amount of M Abs needed to self-block and to cross-block. BT474 cells were treated as described above with saturating concentrations of unlabeled anti-Her-2 M Abs, washed twice, and then treated with various amounts of FITC-labeled anti-Her-2 M Abs. The percentage of cross-blocking was determined by comparing the percentage of positive cells treated with unlabeled anti-Her-2 M Abs followed by FITC-anti-Her-2 M Abs versus the percentage of positive cells treated with FITC-anti-Her-2 M Abs alone. Cross-blocking was considered positive if the binding of the FITC-anti-Her-2 M Abs was reduced by at least 50%.

The isotype of each of the anti-Her-2 M Abs was determined using the ISOSTrip Mouse Monoclonal Antibody Isotyping kit (Boehringer Manheim, Indianapolis, IN) according to the manufacturer’s directions. All of the M Abs were of the IgG1k isotype.

Of these 12 M Abs, three IgG1 M Abs (HER-50, HER-66, and HER-70), which recognize three different epitopes on the EC domain of Her-2, were selected for in vivo evaluation because of their superior ability to inhibit the growth of the Her-2-overexpressing BT474 cell line in vitro.

Growth of BT474 s.c. Tumors in SCID Mice. Male CB.17-SCID mice were used. The sera from all of the mice were screened by ELISA for the presence of mouse immunoglobulin. If serum levels exceeded 10 µg/ml, the mice were considered "leaky" and were not used because tumor-take in these mice is less reliable (26). The mice were then irradiated with 150 cGy and given a single s.c. injection of 4 × 10⁶ BT474 cells/mouse. Tumor nodules were allowed to grow for 10 weeks or until they reached 400 mm³. The diameter of s.c. tumors was measured twice a week using Venier calipers, and the volumes were calculated according to the formula: volume (mm³) = length (mm) × width (mm²).

IHC. s.c. tumors were removed from mice at the end of the experiment after anesthetizing the animals and perfusing them with heparinized saline followed by 4% paraformaldehyde. Mouse tumors were fixed in 10% formalin for 24 h and then embedded in paraffin. Three-µm paraffin sections were prepared at the U. T. Southwestern Pathology Laboratory. Paraffin-embedded tumors were stained with 5 µg/ml affinity-purified polyclonal rabbit antihuman c-erbB-2 antibody (Dako Co., Carpinteria, CA), followed by 2.5 µg/ml biotinylated goat antirabbit IgG (27–29). The same sections were also immunostained with 20 µg/ml HER-50, HER-66, or HER-70 M Abs, or the isotype-matched (negative control) anti-CD25 M Ab, RFT5, followed by 2.5 µg/ml biotinylated horse antimouse IgG. The endothelial cells were immunostained with 5 µg/ml rat antimouse CD31 (PECAM-1), followed by 2.5 µg/ml biotinylated rabbit antirat IgG. Positive reactions were visualized by horseradish peroxidase-conjugated streptavidin-biotin complex and diaminobenzidine. Sections were counterstained with hematoxylin.

ADCC Assays. ADCC assays were performed as previously described (30) with some modifications. The peripheral blood mononuclear cells used as human effector cells were separated from the blood of healthy donors on Ficoll-Hypaque density gradients. Cells were washed once in PBS and then incubated with the green fluorescence cytoplasmic dye, CFSE (Molecular Probes, Inc., Eugene, OR), using 25 µl of a 10-µM solution/10⁶ cells for 7 min at 37°C in a water bath. Murine effector cells were obtained from the spleens of BALB/c mice and were stimulated with concanavalin A (2.5 µg/ml) for 24 h. BT474 (target) cells were harvested, washed, and incubated with different dilutions of either a single anti-Her-2 MAb (HER-50, HER-66, and HER-70), the MAb mixture, Herceptin, or isotype-matched murine or human IgGs (controls). After 30 min at 4°C, excess M Abs were washed out with medium containing 1% FCS plus 0.05% sodium azide. Target cells (BT474) were mixed with either murine or human effector cells at various E:T cell ratios (10:1, 50:1, and 100:1) and incubated for 7 h in a 37°C, 5% CO₂ incubator. PI was used at 50 µg/ml to stain dead cells before analyzing the cells on a Becton Dickinson FACS.

CDC Assays. The CDC assay was similar to the ADCC assay with some modifications (30). For the murine M Abs, mouse serum (Sigma) was used as a source of complement. For Herceptin, human serum isolated from freshly drawn blood was used as one source of complement and mouse serum (Sigma) was used as another. BT474 target cells, treated as described previously, were mixed with either mouse or human sera diluted 1/10, 1/50, or 1/100, and the cells were incubated for 4 h in a 37°C, 5% CO₂ incubator. PI was used at 50 µg/ml to stain dead cells before FACS analysis.
Table 1 Characterization of the anti-Her-2 MAbs

<table>
<thead>
<tr>
<th>MAb</th>
<th>% positive cells</th>
<th>MFI</th>
<th>Relative-binding affinity (× 10^−9 M)</th>
<th>Cross-blocking</th>
</tr>
</thead>
<tbody>
<tr>
<td>HER-50</td>
<td>96.3 ± 2.0</td>
<td>898.7 ± 102.4</td>
<td>0.8 ± 0.5</td>
<td>+</td>
</tr>
<tr>
<td>HER-66</td>
<td>91.7 ± 4.5</td>
<td>1080.7 ± 505.9</td>
<td>3.8 ± 3.2</td>
<td>+</td>
</tr>
<tr>
<td>HER-70</td>
<td>81.5 ± 9.1</td>
<td>2553.8 ± 782.9</td>
<td>13.5 ± 1.5</td>
<td>−</td>
</tr>
<tr>
<td>Herceptin</td>
<td>84.2 ± 3.2</td>
<td>450.4 ± 206.1</td>
<td>0.4 ± 0.1</td>
<td>+</td>
</tr>
</tbody>
</table>

* Cells (1 × 10^6/m) were treated with 1 μg/ml anti-Her-2 MAbs, followed by FITC-labeled goat antimouse immunoglobulin under saturating conditions and analyzed by FACSscan. The percentage of positive cells was plotted versus the MAb concentration to determine the concentration necessary to reach 50% saturation of cells (i.e., the relative binding affinity).

**For the cross-blocking assay, cells were saturated with unlabeled anti-Her-2 MAbs and then treated with different concentrations of the same MAbs labeled with FITC. Cross-blocking was considered positive if the binding of the FITC-anti-Her-2 MAbs was reduced by ≥50%. SDs are based on at least three experiments.

Results

Characterization of the Three Anti-Her-2 MAbs. We have developed a panel of murine anti-Her-2 MAbs that recognize nine different epitopes on the EC domain of Her-2. All of the MAbs were of the IgG1k isotype as determined using the ISOSTRIP Mouse Monoclonal Antibody Isotyping kit. The MAbs were tested for their ability to bind to specific epitopes on the EC domain of Her-2, and the epitopes recognized were determined by cross-blocking (32, 33).

Each MAb and Herceptin could self-block but none could cross-block, suggesting that they recognized four different epitopes on the Her-2 molecule (Table 1). BT474 cells were stained with each of the anti-Her-2 MAbs followed by FITC-labeled goat antimouse immunoglobulin to determine the percentage of positive cells and the density of Her-2 epitopes recognized by the MAbs (MFI). Over 90% of the cells stained positively with HER-50 or HER-66, and >80% of the cells stained positively with HER-70 or Herceptin. However, the epitopes recognized by HER-70 were expressed at the highest density (highest MFI), followed by the epitopes recognized by HER-66 and HER-50. When the relative-binding affinities of all MAbs were determined (by plotting the percentage of positive cells versus the concentration and calculating the concentration required to reach 50% saturation), Herceptin and HER-50 showed the highest relative-binding affinities on BT474 cells. HER-70 had the lowest relative-binding affinity, and HER-66 was intermediary.

Growth Inhibition and Apoptotic Effect of the Anti-Her-2 MAbs on BT474 Cells. We next determined whether the 3 MAbs would inhibit [3H]thymidine incorporation in BT474 cells. The MAbs were incubated for 72 h with BT474 cells, and the levels of [3H]thymidine incorporation in the MAb-treated cells were measured and compared with those of untreated cells to determine the IC50. As shown in Fig. 1 and Table 2, the IC50 of the MAb mixture was decreased 3–200-fold as compared with any one of the three MAbs alone or to Herceptin. Table 2 summarizes the means ± SD of three experiments.

We also determined whether the MAb mixture would induce apoptosis of BT474 cells more effectively than the individual MAbs. Table 2 shows the means ± SD of three experiments. As measured by Annexin-V staining, the MAb mixture...
induced the highest percentage of apoptotic BT474 cells, i.e., 20–27% higher than that induced by individual MAbs and 34% higher than that induced by Herceptin. Cell death, as determined by trypan blue exclusion and PI staining, was similar for the 3 MAbs and Herceptin and marginally superior for the MAb mixture. Levels of apoptosis and necrosis in the untreated controls were negligible. The positive control, sodium azide, induced significant apoptosis. Because the percentage of apoptotic cells induced by the MAbs surpassed the percentage of necrotic cells, it is possible that apoptosis is the major type of cell death induced by the MAbs.

The Therapeutic Effect of Anti-Her-2 MAbs in SCID Mice with s.c. BT474 Tumors. We next determined whether HER-50, HER-66, and HER-70 would inhibit the growth of the BT474 tumor in mice. The in vivo model used to evaluate therapy has been described previously (34); in this model, there is 100% tumor-take and consistent tumor growth after s.c. injection of BT474 cells into preirradiated male SCID mice. Because the growth of BT474 cells is estrogen independent, (35–37) male mice could be used. This is important because estrogen can down-regulate Her-2 expression on tumor cells – (35–37) male mice could be used. This is important because estrogen can down-regulate Her-2 expression on tumor cells and estrogen can down-regulate Her-2 expression on tumor cells (35–37).

Fig. 1   Killing of BT474 cells by the anti-HER-2 MAbs. BT474 cells were treated with varying concentrations of HER-50 (●), HER-66 (○), HER-70 (▲), combination of the three MAbs (■), or Herceptin (▼) for 72 h, then pulsed with [3H]thymidine for 6 h. Growth inhibition was calculated by comparing [3H]thymidine incorporation in treated versus untreated cells. The difference between the IC_{50} of the MAb mixture and the individual MAbs is statistically significant with P<0.05. This is one representative experiment of three performed.

Table 2  A combination of three anti-Her-2 antibodies induces more significant growth inhibition and apoptosis in vitro

<table>
<thead>
<tr>
<th>Antibody</th>
<th>IC_{50} × 10^{-8} (M)</th>
<th>% Annexin-V+ cells</th>
<th>% PI+ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>HER-50</td>
<td>0.3 ± 0.1 d</td>
<td>51.1 ± 11.6</td>
<td>11.7 ± 6.7</td>
</tr>
<tr>
<td>HER-66</td>
<td>7.1 ± 6.0</td>
<td>54.9 ± 2.9</td>
<td>16.3 ± 6.5</td>
</tr>
<tr>
<td>HER-70</td>
<td>20.1 ± 16.2</td>
<td>56.9 ± 13.3</td>
<td>14.2 ± 5.4</td>
</tr>
<tr>
<td>Mixture</td>
<td>0.1 ± 0.04</td>
<td>76.9 ± 11.0</td>
<td>15.8 ± 12.5</td>
</tr>
<tr>
<td>Herceptin</td>
<td>0.3 ± 0.1</td>
<td>43.8 ± 3.4</td>
<td>10.0 ± 8.5</td>
</tr>
<tr>
<td>None</td>
<td>NA</td>
<td>23.0 ± 10.4</td>
<td>4.9 ± 3.4</td>
</tr>
<tr>
<td>NaN3</td>
<td>ND</td>
<td>66.9 ± 9.9</td>
<td>20.5 ± 6.2</td>
</tr>
</tbody>
</table>

a Cells (2.5 × 10⁶/ml) were incubated for 72 h at 37°C with different concentrations of anti-Her-2 MAbs and Herceptin and then pulsed with [3H]thymidine for 6 h. IC_{50} represent the concentration of MAbs required to kill 50% of cells.

b,c Cells (2.5 × 10⁵/ml) were incubated 4 h at 37°C with 100 μg/ml anti-Her-2 MAbs and Herceptin and then stained with Annexin-V FITC plus PI (50 μg/ml) and analyzed by FACScan. The difference between the IC_{50} of mixture versus HER-66 and HER-70 is statistically significant with P<0.027. The difference between the % Annexin-V+ cells in each treatment group versus untreated control is statistically significant with P<0.05. The difference between the % PI+ cells in each treatment group versus untreated control is statistically significant with P<0.001.

d SDs are based on three experiments carried out.

NA, not applicable, because the untreated control values were taken as 100%; ND, not done.

Positive control.

of ¹²⁵I-HER-66 into both normal and s.c tumor-bearing SCID mice. We observed that 1.5% of the injected dose was taken up by the s.c. breast tumor nodule as compared with 0.7% by the kidneys and liver, 0.4% by the lungs, and 0.09% by the spleen (data not shown). The MAb persisted at the tumor site for at least 4 days.

Therefore, SCID mice with advanced s.c. BT474 tumors measuring 400 mm³ were then treated with 15 μg/g mouse of the individual anti-Her-2 MAbs, the MAb mixture, or Herceptin. The MAbs were administered as three i.p. injections at 3-week intervals.

As shown in Fig. 2A, mice treated with the MAb mixture showed continuous regression of tumor nodules starting after the first course of treatment. After the third course, tumor nodules became nonpalpable for a total of 3 weeks. Individual MAbs induced 75–90% reductions in tumor volume after 2 weeks, and these reductions were maintained over the next 4.5 weeks, followed by relapse. Herceptin was inefficient initially, then induced a 40% reduction in tumor volume after the third week and progressively reduced tumor volumes to 60% of their original size in the following 3.5 weeks, followed by relapse. The reasons for this delay are unclear but may be related to poor ADC/CDC initially, followed by delayed antitumor activity. Fig. 2B shows that the MAb mixture also prolonged the survival of SCID/BT474 mice more effectively than either the individual MAbs or Herceptin. Thus, 100% of mice treated with the MAb mixture survived for 7 weeks after the first injection, whereas 40–60% of mice treated with individual MAbs and 5% of the mice treated with Herceptin survived for the same length of time. Untreated mice were sacrificed when tumor burdens exceeded 1000 mm³ volume or 10% of their total body weight. When mice with smaller, 200 mm³ tumors were treated
with the MAb mixture, tumors became nonpalpable 2 weeks after a single dose (data not shown). Remissions were sustained for 4 weeks and all of the mice treated with the mixture survived for 7 weeks after treatment. None of the individual MAbs or Herceptin induced 100% inhibition of s.c. tumor growth, nor did they prolong survival of mice as long as the MAb mixture. Histopathological examination indicated that none of the treated or untreated mice had metastases in their lungs, livers, spleens, or kidneys (data not shown). These results confirm earlier findings that metastases can only be detected when s.c. tumors are >800 mm$^3$ (34). In all treated animals, tumors resumed growing 2–3 weeks after the last treatment. However, the regressions induced by the MAb mixture generally lasted 1 week longer.

These experiments demonstrate that the MAb mixture is more effective in treating larger tumors than either the individual MAbs alone or Herceptin. When smaller tumors were treated, significant growth inhibition effect was achieved by only one dose of the mixture and was more rapid and long lived.

The Effects of MAb Treatment on the Expression of Her-2 Epitopes on Surviving Tumor Cells. One mechanism by which anti-Her-2 MAbs act is to induce receptor down-modulation (40–42). Therefore, we determined whether the tumor cells which grew back after therapy was discontinued, lacked the Her-2 epitopes recognized by the MAbs used for treatment. Tumors were removed from mice at the end of the experiment, fixed in 10% formalin, embedded in paraffin, and immunostained with HER-50, HER-70, or HER-66 MAbs, the isotype-matched anti-CD25 antibody, RFT5 (negative control), or the anti-c-erbB-2 polyclonal antibody (Dako Co.). As shown in Fig. 3, tumor nodules removed from relapsed mice expressed the same levels of Her-2 as the original tumor. This indicates that tumor cells that grew back either had not down-regulated Her-2 or that down-regulation was reversible. These findings suggest that the relapsed tumors do not lack the targeted epitope, but rather escape killing by the MAbs for other reasons, e.g., inaccessibility, mutations in signaling pathways, and so on.

The Ability of the Anti-Her-2 MAbs to Mediate ADCC and CDC. Although the MAb mixture was highly effective at inhibiting cell growth in vivo, it has been documented that in vivo the Fc portion of MAbs can be of major importance in efficacy (43–45). Furthermore, the binding of MAbs to three different epitopes on Her-2 might be more efficient in mediating effector functions because of higher concentrations of total antibody bound. This should contribute to a superior performance of the MAb mixture in vivo. To explore this possibility, we compared the effector functions of the individual MAbs to those of the MAb mixture in vitro. As shown in Fig. 4A, the individual MAbs induced 10–20% lysis of target cells, whereas the MAb mixture induced 30% lysis at an E:T cell ratio of 100:1. Herceptin mediated potent ADCC in the presence of human peripheral blood mononuclear cells at an E:T cell ratio of 100:1, inducing up to 50% lysis of target cells. However, Herceptin mediated only 10% lysis in the presence of murine effector cells (data not shown), at least partially explaining its inferior activity in mice as compared with the murine MAbs. As shown in Fig. 4B, the MAbs also fixed complement, resulting in dose-dependent lysis of the target cells. At the same dilution of complement, the MAb mixture induced 2-fold more lysis than HER-50 or HER-66 alone and 10% more lysis than Herceptin or HER-70. Herceptin induced only 10% lysis in the presence of mouse serum, again partially explaining why it was less effective in mice. The isotype-matched negative controls did not mediate either ADCC or CDC.

The Effect of Anti-Her-2 MAbs Treatment on VEGF Secretion by Tumor Cells in Vitro. As described by others (15), treatment with anti-Her-2 MAbs may also exert an antiangiogenic effect in vivo by down-regulating the production of one or more proangiogenic factors produced by the tumor cells. We have documented the presence of newly formed vessels in the s.c. breast tumor nodules by immunostaining tumors with MAbs that react with the endothelial cell marker PECAM-1 (CD31; data not shown). All tumors contain a high density of vessels, which are immunoreactive with the anti-CD31 anti-

Fig. 2  BT474 cells are killed most effectively in vivo by the MAb mixture. A, preirradiated male SCID mice with 400 mm$^3$ s.c. BT474 breast tumors were injected i.p. with three equal doses of 300 μg (15 μg/g mouse) 10 weeks after tumor inoculation on days 0, 24, and 48. The following treatments were administered to groups of five mice: HER-50 (●); HER-66 (○); HER-70 (▲); combination of the three MAbs (■); Herceptin (▼); and control (injected with saline; □). Tumors were measured twice a week using calipers, and the mean tumor volume (mm$^3$) ± SD for the five mice in each group was plotted. This represents one experiment of three carried out. The difference between the effects of each treatment versus the control, and between the MAb mixture and each individual MAb are significant with Ps < 0.00715 and < 0.05, respectively. B, one hundred percent of mice treated with the combination of three MAbs survived 7 weeks after the first dose injected. A total of 40–60% of mice treated with individual MAbs and 5% of Herceptin-treated mice survived after 7 weeks when the experiment was terminated. Animals were euthanized when tumor burden exceeded 1000 mm$^3$ or 10% of total body weight.
body. These results suggest that angiogenesis is involved in the growth of s.c. BT474 tumors.

Therefore, we used a competitive ELISA assay to determine whether the three anti-Her-2 MAbs could suppress VEGF production by BT474 cells in vitro. We measured VEGF levels in cells treated with either the individual MAbs, the MAb mixture, or Herceptin. As shown in Fig. 5, untreated BT474 cells secreted ~50 ng VEGF/ml/5 x 10^5 cells over 48 h. Increasing concentrations of the anti-Her-2 MAbs induced a dose-dependent decrease in VEGF production. The MAb mixture completely inhibited VEGF production at a concentration of 1 μg/ml. HER-50 and Herceptin induced appreciable but not complete inhibition of VEGF secretion, whereas HER-66 and HER-70 had marginal activity at the same concentration. These results indicate that all of the MAbs block the secretion of VEGF but that the MAb mixture is superior.

Discussion

In this study, we have compared the antitumor activity of three new anti-Her-2 MAbs (HER-50, HER-66, and HER-70) alone, as a MAb mixture, and versus Herceptin. Initially, 12 MAbs recognizing nine epitopes on Her-2 were generated and tested for their ability to bind to specific epitopes on the EC domain of Her-2. The three MAbs described in this study were selected based on their potent inhibition of the growth of BT474 cells in vitro. Herceptin is the fully humanized variant of the anti-Her-2 MAb, 4D5, produced by Genentech and was used as a reference MAb. Herceptin has been approved by the Food and Drug Administration for treating women with Her-2-overexpressing breast carcinomas (24, 25, 46–48). Currently, Herceptin is being used in patients in several combinatorial regimens.

We (49, 50) and others (51–53) have previously demonstrated that hypercrosslinking of cell surface molecules on lymphoma cells is important for negative signaling. Hypercrosslinking can be achieved by homodimerizing individual MAbs or by mixing high affinity MAbs directed against different epitopes on the same molecule. Therefore, we determined whether a combination of three anti-Her-2 MAbs that would be predicted to hypercrosslink Her-2 more effectively on breast cancer cells would have more potent cytotoxic activity than the individual MAbs. The major findings to emerge from this study are as follows: (a) as compared with individual MAbs (including Herceptin), in vivo therapy with the MAb mixture induced more rapid tumor regressions, increased the time to relapse, and prolonged the survival of the treated mice; and (b) as compared with individual MAbs, the MAb mixture showed improved Fc-mediated effector functions, improved blocking of VEGF secretion, and improved direct antiproliferative and proapoptotic effects.

Our in vivo results indicate that in SCID mice with 400 mm^3 BT474 xenografts, the MAb mixture, as compared with the individual MAbs, induced complete but transient regressions of tumor nodules and also prolonged both survival and the time to relapse. As determined by IHC, BT474 tumor nodules removed from relapsed mice expressed the same levels of Her-2 epitopes recognized by the MAbs used for treatment as the original tumor. Hence, relapses were not due to the lack of targetable epitopes but rather to escape from killing by the MAbs for other reasons, e.g., mutations in signaling pathways, reversible down-regulation of Her-2 epitopes, inaccessibility, and so on. It is possible that cures might have been achieved with additional or larger doses.

To determine why the MAb mixture was superior to the individual MAbs in vivo, we compared their mechanisms of action (54). In three [3H]thymidine incorporation experiments, the IC50 of the MAb mixture was decreased as compared with the individual MAbs, demonstrating that it had superior anti-
growth activity. The mixture induced apoptosis in BT474 cells more effectively than the individual MAbs.

It has been reported previously that anti-Her-2 MAbs can signal tumor cells to undergo growth arrest or cell death by competitive binding to growth factor receptors, inhibition of ligand binding, and suppression of transcription of growth factors (54–56). MAbs that target growth factors or cellular growth factor receptors that are required for tumor cell survival mediate cytostatic or cytotoxic effects (57). Antiepidermal growth factor receptor MAbs have antitumor activity and effectively induce cell cycle arrest or apoptosis in Her-2\textsuperscript{+} tumor cells (48, 58, 59). Anti-Her-2 MAbs induce down-modulation of Her-2 epitopes and prevent the growth of tumors in vivo (40–42, 60, 61). Anti-Her-2 MAbs can also kill tumor cells indirectly through immunological mechanisms, including ADCC and CDC (62–65), and these effects are dependent upon the immunoglobulin isotype of the MAb. With regard to murine MAbs, IgG\textsubscript{2a} and IgG\textsubscript{3} MAbs are the most effective at mediating ADCC and CDC (66, 67). Anti-Her-2 MAbs might also inhibit angiogenesis by blocking Her-2-mediated signal transduction pathways that upregulate the expression of proangiogenic factors such as VEGF (10–13).

Regarding the in vitro efficacy of murine anti-Her-2 MAbs, the 4D5 MAb is one of the most potent growth inhibitory anti-Her-2 MAbs (68). Treatment of breast cancer cells with 4D5 partially blocks Heregulin activation of Her-2-Her-3 complexes, as measured by receptor phosphorylation assays (69). 4D5 exhibited significant antiproliferative activity in vitro against cell lines that overexpress Her-2 (70) and induced dose-dependent cell cycle arrest with maximal inhibitory activity occurring at MAb concentrations > 1 µg/ml (71). 4D5 sensitized Her-2-overexpressing cell lines to tumor necrosis factor α (64) and restored adhesion molecules E-cadherin and α2 integrin to normal levels, turning off the metastatic process and malignant progression (72). The fully humanized variant of 4D5, Herceptin, binds to the EC domain of Her-2 with an affinity (K\textsubscript{d}) of 0.1 nmol/liter, which is 3-fold greater than that of 4D5 (73).

In several models of MAb therapy, MAbs have demonstrated antigrowth activity and the ability to induce apoptosis (57, 69, 74–77). There is also evidence that the superior antitumor activity of some MAbs in vivo can be attributable to enhanced negative signaling and that signaling can be enhanced by hypercrosslinking using homodimers (49, 51) or mixture of antibodies (78). Wolff et al. (51) previously reported that homodimers of a breast tumor-reactive MAb, Chi BR96, were...
10-fold more effective both in vitro and in vivo. Homodimers also showed improved antitumor activity as compared with the monomers in nude mice bearing human lung adenocarcinoma xenografts (51). In this regard, genetically engineered multimeric IgG constructs offer the advantage of increased therapeutic potential. As described previously, homodimeric IgG was 100-fold more potent in mediating CDC and ADCC than monomers (52, 53). Homodimers of Rituxan (chimeric anti-CD20 MAb) were superior in signaling G$_{s}$/G$_{i}$ arrest and in inducing apoptosis in several B-lymphoma cell lines in vitro. Treatment with homodimers, compared with monomers, also rendered tumor cell lines more sensitive to chemotherapeutic agents and synergized with an anti-CD22-immunotoxin in vitro (49, 50). Similar results have been described for a mixture of immunotoxins that target more than one surface antigen, which have demonstrated better in vivo activity in experimental animals than single immunotoxins (79, 80).

Although the inhibition of cell growth is often important in vitro, the effector functions of MAbs can also be equally or more important in vivo (62–65). In addition to superior apoptotic activity, the MAb mixture induced a 3-fold increase in cell lysis in our in vitro ADCC assays as compared with each of the individual MAbs. Hence, improved ADCC may also be involved in the superior performance of the MAb mixture in vivo.

The inferior activity of Herceptin versus the murine MAbs in vivo is not surprising and in no way predicts that it would have less activity in humans. Thus, the effectiveness of a MAb depends upon whether the Fc portion of the antibody is of the same species as the one being treated (81, 82). Hence, the Fc portion of murine MAbs can fix mouse murine complement and interact efficiently with FcRs on mouse immune effector cells. In contrast, the binding of a human IgG1 to FcγRIII on murine natural killer cells, macrophages, or neutrophils, as well as to mouse complement, is relatively weak (81). Our data confirm these results, indicating that although Herceptin mediated ADCC/CDC works well in vitro with human immune effector cells/serum, it works poorly with murine immune effector cells and serum. Hence, its activity in mice with human tumor xenografts gives a poor estimate of its potency in humans.

With regard to in vivo activity in mice, the antitumor activity of Herceptin relative to a control human IgG1 was compared with that of its murine parent MAb, 4D5 (83). There was a 10-fold decrease in tumor size using 4D5 at 25 mg/kg and Herceptin at 100 mg/kg. Thus, the murine MAb was a more effective antitumor agent in mice than Herceptin. Our in vivo results also correlate with previously reported data on Herceptin where dose-dependent antitumor activity using doses ranging from 0.1 to 1 mg/kg and a plateau at doses > 1 mg/kg occurred in nude mice bearing BT474 breast carcinoma xenografts (83). Pegram et al. (71) described dose-dependent antitumor activity with 3–100 mg/kg in athymic mice bearing MCF7 breast carcinoma xenografts. BT474 xenografts are therefore more sensitive to Herceptin treatment than the MCF7 xenografts.

In addition to inducing apoptosis and mediating effector function, anti-Her-2 MAbs could also exert antitumor activity by inhibiting angiogenesis. Angiogenesis is critical for the growth and survival of solid tumors (84, 85). VEGF is one of the most important mediators of tumor angiogenesis (86) and is also an important factor required for the growth of BT474 tumors (15). It has been demonstrated that the treatment of BT474 tumor cells with anti-Her-2 MAbs decreases VEGF production because of the suppression of VEGF gene expression (15). Suppression of angiogenesis enhanced the activity of anti-Her-2 MAb therapy in vivo (15). Our results are in agreement with these findings, suggesting that angiogenesis is involved in the growth of s.c. BT474 tumors, and that each of the three murine anti-Her-2 MAbs, the MAb mixture, and/or Herceptin all induced a dose-dependent decrease in VEGF production by BT474 cells in vitro. The MAb mixture was the most effective and completely inhibited VEGF secretion at a concentration of 1 μg/ml HER-50 and Herceptin induced appreciable but not complete inhibition of VEGF secretion, whereas HER-66 and HER-70 inhibition was marginal at the same concentration. These results indicate that all of the MAbs block the secretion of VEGF but that the MAb mixture is superior.

In summary, the results presented in this study suggest that the murine anti-Her-2 MAb mixture, as compared with individual murine MAbs, has improved antitumor activity against Her-2-overexpressing breast carcinoma cell line BT474 in vivo and in vitro. This superior activity is probably attributable to a combination of factors, including enhanced antiproliferative and antiapoptotic effects, improved Fc-mediated effector functions, and an increased ability to inhibit VEGF secretion. Although it is not possible to make any valid comparisons between humanized Herceptin and our murine MAbs in mice, our results suggest that a mixture of humanized anti-Her-2 MAbs might be superior to Herceptin alone in humans.

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**References**

Targeting Multiple Her-2 Epitopes with MAbs


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