A Comparison of the *in Vitro* and *in Vivo* Activities of IgG and F(ab′)$_2$ Fragments of a Mixture of Three Monoclonal Anti-Her-2 Antibodies

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ABSTRACT

Purpose: We have demonstrated previously that a mixture of three anti-Her-2 monoclonal antibodies (MAbs) that bind to different epitopes on the extracellular domain of Her-2 expressed on a human breast cancer cell line has more potent antitumor activity than the individual MAbs both *in vitro* and in xenografted severe combined immunodeficient mice. Because the activity of Herceptin is Fc dependent, we determined whether this would also be the case when a mixture of these three anti-Her-2 MAbs was used.

Experimental Design: IgG and highly purified F(ab′)$_2$ fragments of the anti-Her-2 MAbs and Herceptin were prepared and evaluated for their ability to induce cell death, inhibit vascular endothelial growth factor secretion, and mediate antibody-dependent cellular cytotoxicity and complement-mediated cytotoxicity *in vitro*. They were also compared for their abilities to induce regression of large BT474 tumors in severe combined immunodeficient mice.

Results: All of the F(ab′)$_2$ fragments were >95% pure and, as expected, did not mediate antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity *in vitro*. The *in vitro* antiproliferative and proapoptotic effects of the IgGs and F(ab′)$_2$ fragments were similar. In contrast, the IgGs had significant antitumor activity *in vivo*, whereas their F(ab′)$_2$ fragments were only marginally effective even at 5-fold higher doses to offset their shorter half-lives.

Conclusions: These results confirm the importance of the Fc portion of Herceptin for optimal *in vivo* activity and demonstrate that even a mixture of three anti-Her-2 MAbs that are highly effective at inducing cell death *in vitro* requires Fc-mediated effector function for optimal *in vivo* activity.

INTRODUCTION

The erbB family of tyrosine kinase transmembrane receptors is involved in the regulation of various physiological processes including cell growth, differentiation, cell-cell interactions, and cytokine signaling (1, 2). Overexpression of the erbB-2 (Her-2) receptor on various human cancers including breast, ovarian, prostate, gastric, lung, bladder, and kidney carcinomas (3) is generally associated with enhanced metastatic potential and a poor prognosis (4). The levels of Her-2 expression correlate with the degree of breast tumor differentiation, estrogen receptor status, and clinical outcome (5). One of the mechanisms responsible for the aggressive behavior of Her-2$^+$ tumor cells is the up-regulation of growth factor-mediated tumor angiogenesis, which correlates with the overexpression of Her-2 (6–9). A proangiogenic factor, vascular endothelial growth factor (VEGF), is down-regulated by some anti-Her-2 monoclonal antibodies (MAbs), resulting in the inhibition of tumor growth (10, 11). Because Her-2 is expressed at lower levels on cells in many normal tissues, it makes an attractive target for immunotherapy (12). This was first demonstrated in 1986 by Drebin *et al.* (13) using a rat neuroblastoma model.

Many MAbs raised against Her-2 inhibit the *in vitro* growth of Her-2-overexpressing tumor cells by blocking signaling pathways, activating apoptosis, inhibiting VEGF secretion or activity, and mediating effector cell- and complement-mediated cytotoxicity (14). The humanized anti-Her-2 MAb, 4D5 (Herceptin or trastuzumab), has been approved by the United States Food and Drug Administration for human use and has increased the survival of relapsed patients with Her-2-overexpressing breast tumors (15, 16). Murine MAbs and Herceptin engage both activating FcγRIII as well as inhibitory FcγRIIb receptors on immune cells, required for effective antitumor cytotoxicity *in vivo*. When Fc binding is reduced or eliminated, Herceptin loses virtually all of its antitumor activity *in vivo* (17); hence, Fc-dependent antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) are critical for *in vivo* efficacy.

We have demonstrated previously that the antigrowth activity of a mixture of anti-Her-2 MAbs that recognize different epitopes on its extracellular domain was superior to that of the individual antibodies both *in vitro* and *in vivo* (18). This raised the possibility that when such mixtures were used, "negative signaling" might be more important than Fc-mediated effector function. To explore this hypothesis, we have compared the antitumor activity of the IgG and F(ab′)$_2$ fragments of a mixture of three anti-Her-2 IgGs (HER-50, HER-66, and HER-70) and the IgG and F(ab′)$_2$ fragment of Herceptin. We have determined that the IgGs and their F(ab′)$_2$ fragments have equal antitumor activity *in vitro*. In addition, the mixture of the three F(ab′)$_2$ fragments is superior to each one alone. As expected, F(ab′)$_2$ fragments did not mediate ADCC or CDC *in vitro*. In contrast,

as compared with the similar activity of IgG and F(ab′)₂ fragments *in vitro*, only the IgGs were highly effective *in vivo*. Indeed, 5-fold higher concentrations of the mixture of F(ab′)₂ had only very modest antitumor activity.

MATERIALS AND METHODS

Cells. The human breast cancer cell line BT474 (American Type Culture Collection, Manassas, VA) was maintained in monolayer culture in MEM (GIBCO BRL, Grand Island, NY) supplemented with 1% vitamins, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 1% HEPES buffer (GIBCO BRL), and 10% heat-inactivated FCS (Hyclone, Logan, UT). Cultures were grown at 37°C and 5% CO_2, 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 (GIBCO BRL) at room temperature and washed twice in complete medium.

MAbs. The mouse anti-Her-2 MAbs were generated in our laboratory as described previously (19) by s.c. immunization of BALB/c mice with the extracellular domain of Her-2. The MAbs were affinity purified on protein G-Sepharose (Pharmacia, Piscataway, NJ) and further characterized.

Preparation of F(ab′)₂ Fragments. The selected MAbs were brought to 5 mg/ml and dialyzed overnight *versus* 0.1 M acetate buffer (pH 3.8). Insoluble pepsin (Sigma) was added, and the digestion was performed at 37°C for 7 h with rotation. SDS-PAGE analysis showed that >95% of the IgG was digested to F(ab′)₂ and peptides during this period of time. The digestion was stopped by adding 0.1 volume of 2 M Tris-HCl buffer (pH 8.2) and filtration through a 0.22-μm Millex filter (Millipore Corp., Bedford, MA). The digests were chromatographed twice on a Sepharose SpA column equilibrated with PBS (pH 7.4). The flow-through was dialyzed overnight against 50 mM phosphate buffer with 150 mM NaCl [PBS (pH 7.4)]. After concentration, the protein solutions were filtered through a 0.22-μm Millex filter and stored at 4°C until used.

The purity of all preparations was determined by SDS-PAGE under reducing and nonreducing conditions (18). Gel electrophoresis was performed using the PhastGel System (Pharmacia) on a continuous 4–15% gradient polyacrylamide gel gradient with 2% cross-linking. Gels were stained by Coomassie Blue with an aqueous solution containing of 0.1% PhastGel Blue R (Pharmacia), 10% acetic acid, and 30% methanol and destained with a solution consisting of 10% acetic acid and 30% methanol. The following molecular mass standards (Sigma) were used for comparison: 212 kDa; 170 kDa; 116 kDa; 76 kDa; and 53 kDa.

The purity of F(ab′)₂ fraction was determined by scanning the gel using the Pharmacia LKB UltroScan XL gel scan, and results were interpreted using the gel scan software (Pharmacia).

Characterization of the F(ab′)₂ Fragments. The epitope specificity of the three MAbs had been determined previously by using direct immunofluorescence blocking assays (19). The three MAbs and their F(ab′)₂ fragments were characterized for binding to BT474 cells using direct immunofluorescence assays. The percentage of positive cells was plotted *versus* the molar concentration of F(ab′)₂ and IgG to determine the concentration necessary to reach 50% saturation of cells (*i.e.*, the relative binding affinity).

Pharmacokinetics of IgGs and F(ab′)₂ Fragments of HER-50 in Severe Combined Immunodeficient (SCID) Mice. One hundred μg of each protein were radiolabeled with 1 mCi of Na¹²⁵I (Amersham Biosciences, Piscataway, NJ) using the Iodo-Gen procedure (20). The free ¹²⁵I was removed by centrifugation on MicroSpin (Pharmacia). Each protein was resuspended in 300 μl of 1% BSA in PBS, and 100 μl were injected i.p. (100 μl with $0.3–1 \times 10^7$ cpm at a dose of 33.3 μg/animal). Six male SCID mice (Taconic, Germantown, NY) were given sweetened drinking water with 0.05% Lugol's solution 24 h before injection and throughout the entire period of the experiment. The whole body radioactivity was measured immediately after injection and then measured on a daily basis for 8 days using the AtomLab 100 Dose Calibrator (Atom Product Corp.). The results were expressed relative to the amount of whole body radioactivity present immediately after injection (percentage of initial radioactivity).

The pharmacokinetic parameters were determined using a noncompartmental model with the PKCALC program (21). These included the following parameters: $t_{1/2}$ (in h), half-life, AUC (in μg-h/ml), area under the curve; MRT (in h), mean residence time; and FCR (in day⁻¹), fractional catabolic rate.

Biodistribution of F(ab′)₂ Fragments in Tumor-Bearing SCID Mice. Radiolabeling with ¹²⁵I was carried out according to the same Iodo-Gen procedure (22). After removal of free iodine by centrifugation on MicroSpin columns, each radiolabeled protein was mixed with the same cold protein at a final concentration of 1 mg/ml, and 100 μl were injected i.p. in three SCID male mice (4.8×10^8 cpm in 33.3 μg/animal). Tumor, blood, and organs were harvested after perfusing mice with 10 units/ml heparinized saline. Solid organs were homogenized by grinding and precipitated with 10% trichloracetic acid. Levels of radioactivity were measured in a gamma counter at 24, 48, and 72 h after injection. The results were expressed relative to the injected dose and organ weight or ml of serum (percentage of initial injected dose/g tissue or ml of serum).

ADCC Assays. ADCC assays were performed as described previously (22), with modifications. Murine effector cells were obtained from the spleens of BALB/c mice and stimulated with Concanavalin A (2.5 μg/10⁶ cells) for 24 h. The peripheral blood mononuclear cells used as human effector cells were separated from the blood of healthy donors on Ficoll-Hypaque (Amersham Biosciences) density gradients. Cells were washed once in PBS and then incubated with the green fluorescence cytoplasmic dye 5-(and-6)-carboxyfluorescein diacetate, succinimidylester (Molecular Probes, Inc, Eugene, OR), using 25 μl of a 10 μM solution/10⁶ cells for 7 min in a 37°C water bath. BT474 (target) cells were harvested, washed and coated with 1 μg/10⁵ cells of either a mixture of F(ab′)₂ fragments of HER-50, HER-66, or HER-70 or the same molar amount of the mixture of the HER-50, HER-66, or HER-70 IgG. Isotype-matched IgG or F(ab′)₂ fragments of RFT5, the antihuman CD25 MAb, were used at the same molar concentrations. Ten mg/ml NaN_3 was used as the positive control. After 30 min at 4°C, excess proteins were washed out with medium containing 1% FCS plus 0.05% NaN_3. 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 humidified 37°C, 5% CO_2 incubator. Propidium iodide (PI) was used

at 50 μg/ml to stain dead cells before analyzing the cells on a FACSscan.

CDC Assays. The CDC assays were similar to the ADCC assays with modifications (22). Mouse serum (Sigma) and human serum isolated from freshly drawn blood were used as sources of complement.

Measurement of VEGF in the Supernatants of BT474 Cells by ELISA. A total of 5×10^5 BT474 tumor cells/well were plated in 24-well plates (Corning, Inc., Corning, NY). Cells were allowed to adhere overnight and then treated with different concentrations of either a mixture of F(ab′)₂ fragments of HER-50, HER-66, and HER-70 or the mixture of the three IgGs at the same molar concentrations. IgG or F(ab′)₂ fragments of the isotype-matched RFT5 were also used at the same molar concentrations. Ten mg/ml NaN₃ was used as the positive control. Aliquots of cells were removed at various time points, centrifuged, and the supernatants filter sterilized. VEGF levels were measured using an ELISA kit (Cytimmune Sciences, Inc.). The amount of VEGF present in the supernatants was extrapolated from the VEGF standard curve and expressed in ng/ml. The levels of VEGF that can be detected in this assay ranged from 0.195 to 200 ng/ml.

In Vitro Growth Inhibition Assay of BT474 Cells. BT474 cells (100 μl/well), at 2.5×10^6 cells/ml in 10% MEM, were plated in triplicate in 96-well microtiter plates (Corning, Inc.) and allowed to adhere overnight. The cells were then treated with 100 μl of different concentrations of a mixture of IgG or F(ab′)₂ fragments of HER-50, HER-66, and HER-70 at the same molar concentrations. IgG or F(ab′)₂ fragments of RFT5 were used as controls. Nine wells of untreated cells were included in each plate, and cells treated with 10 mg/ml NaN₃ were used as the positive control. The plates were incubated for 72 h at 37°C in a 5% CO₂ incubator and then pulsed for 6 h with 1 μCi/well [³H]thymidine (Amersham Biosciences). Inhibition of growth was determined by plotting the percentage of [³H]thymidine incorporation compared with that of untreated cells, which was taken as 100%. The concentrations that inhibited [³H]thymidine incorporation by 50% (IC₅₀) were compared.

Annexin-V Assay. This assay measures early events in apoptosis (23). BT474 cells were plated at 2.5×10^5 cells/ml in 24-well plates, allowed to adhere overnight, and treated with 100 μg/ml of the combination of anti-Her-2 F(ab′)₂ fragments or the equivalent molar concentrations of IgGs, using amounts determined in preliminary [³H]thymidine incorporation experiments to inhibit the growth of BT474 cells by 50%. Untreated cells were used as negative controls, and 10 mg/ml NaN₃ was used as a positive control. After a 4-h incubation, the cells were harvested by trypsinization, washed, and stained with FITC-annexin-V and PI according to the manufacturer's instructions (Biosource International, Camarillo, CA). Fluorescence-activated cell-sorting analysis was used to quantify annexin⁺ (apoptotic) cells and PI⁺ (necrotic) cells.

Growth of Subcutaneous BT474 Tumors in SCID Mice. One-month-old 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, animals were considered "leaky" and were not used (24). The mice were then irradiated with 150 cGy and given a single s.c. injection of 4×10^6 BT474 cells/mouse. Tumor nodules were allowed to grow for 10 weeks or until they reached 200 mm³.
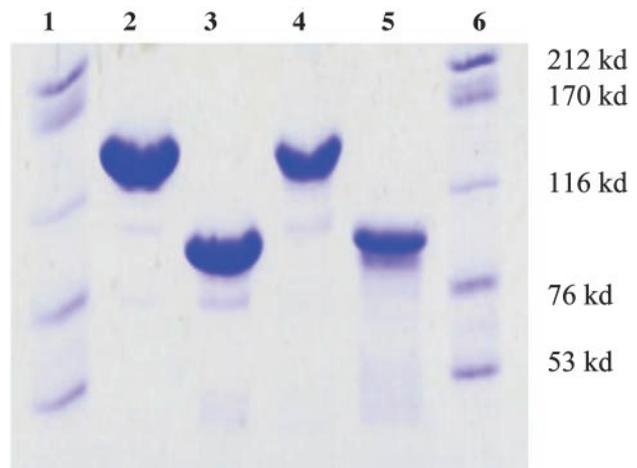
Fig. 1 SDS-PAGE of the purified F(ab′)₂ preparations of the anti-Her-2 monoclonal antibodies. The mixture of F(ab′)₂ preparations was analyzed by SDS-PAGE under nonreducing conditions using the Phast-Gel System. Gels were stained with Coomassie Blue. _Lanes 1_ and _6,_ standard high molecular mass protein markers with molecular weights of 212, 170, 116, 76, and 53 kDa; _Lane 2,_ Herceptin, IgG; _Lane 3,_ F(ab′)₂ fragments of Herceptin; _Lane 4,_ a mixture of HER-50, HER-66, and HER-70 IgGs; _Lane 5,_ mixtures of F(ab′)₂ fragments of HER-50, HER-66, and HER-70. F(ab′)₂ fragments have a molecular mass of ~100 kDa, whereas the whole IgG is ~150 kDa. Scanning the gel by using the Pharmacia LKB UltroScan XL indicated that the F(ab′)₂ fragments of the mixture F(ab′)₂ and Herceptin F(ab′)₂ preparations tested were >95% pure.

The diameters of s.c. tumors were measured twice a week using Venier calipers, and the volumes were calculated according to the following formula: volume (mm³) = [length × (width)²]/2.

Treatment of SCID/BT474 Mice with a Mixture of HER-50, HER-66, and HER-70 IgG or F(ab′)₂ Fragments. One group of five preirradiated SCID mice with 200-mm³ s.c. BT474 breast tumors grown for 10 weeks each received one i.p. injection of 75 μg/g body weight of a mixture of F(ab′)₂ fragments of HER-50, HER-66, and HER-70. Another group of five mice each received one i.p. injection of 15 μg/g mouse of a mixture of the three IgG MAbs (HER-50, HER-66, and HER-70). One group of five mice each received one i.p. injection of the isotype-matched IgG or its F(ab′)₂ (RFT5 anti-CD25; controls). IgG and F(ab′)₂ fragments of Herceptin were injected at doses of 15 and 75 μg/g mouse, respectively. Five control mice received injection with saline. Tumors were measured twice a week using calipers, and the mean tumor volume (mm³) ± SD for the five mice in each group was calculated according to the same formula described above. Animals were euthanized when the tumor burden exceeded 1500 mm³ or 10% of total body weight, in accordance with our Animal Resource Center guidelines.

RESULTS

Characterization of Anti-Her-2 F(ab′)₂ Fragments and IgGs. The isotypes of the anti-Her-2 MAbs were determined previously to be IgG1κ (19). The anti-Her-2 MAbs recognized nonoverlapping epitopes on the extracellular domain of Her-2 (25, 26).

Fig. 1 shows the purity of antibody fragments as deter-

Table 1 Characterization of the anti-Her-2 F(ab′)$_2$ fragments and IgGs

	Direct immunofluorescence			Pharmacokinetics parameters in SCID mice[c]			
Antibody	Relative binding affinity (×10^{-9} M)[a]	% Positive cells (100 μg/10^5 cells)	MFI[b]	$t_{1/2}$ (h)	AUC (μg-h/ml)	MRT (h)	FCR (day^{-1})
F(ab′)$_2$							
HER-50	0.23 ± 0.12	97.3 ± 1.6	181.3 ± 24.2	25.6 ± 20.9	1456.8 ± 9.6	91.8 ± 0.1	1.54 ± 0.035
HER-66	5.88 ± 9.88	97.1 ± 1.5	155.1 ± 25.8	N/D	N/D	N/D	N/D
HER-70	2333.3 ± 1247.2	90.53 ± 1.95	52.1 ± 19.4	N/D	N/D	N/D	N/D
Herceptin	0.5 ± 0.2	97.48 ± 0.54	157.7 ± 12.4	N/D	N/D	N/D	N/D
IgG1							
HER-50	0.1 ± 0[d]	93.37 ± 4.2	160.9 ± 51.9	149.8 ± 100.9	30847.3 ± 950.9	222.9 ± 14.9	0.075 ± 0.004
HER-66	6.07 ± 8.43	96.83 ± 1.44	209.2 ± 61.9	N/D	N/D	N/D	N/D
HER-70	1433.3 ± 1144.1	82.15 ± 2.25	69.0 ± 52.9	N/D	N/D	N/D	N/D
Herceptin	0.1 ± 0	97.6 ± 0.85	212.9 ± 64.0	N/D	N/D	N/D	N/D

[a] Cells (10^5 cells/ml) were treated with different concentrations of FITC-anti-Her-2 F(ab′)$_2$ and IgGs, followed by FACScan analysis. The percentage of positive cells was plotted *versus* the F(ab′)$_2$ or IgG concentrations to determine the concentration necessary to reach 50% saturation of cells (*i.e.,* the relative binding affinity).

[b] MFI, mean fluorescence intensity; SCID, severe combined immunodeficient; $t_{1/2}$ (h), half-life; AUC (μg-h/ml), area under the curve; MRT (h), mean residence time; FCR (day^{-1}), fractional catabolic rate; N/D, not done.

[c] The results are expressed as percentage of initial radioactivity relative to the amount of whole body radioactivity after i.p. injection of 100 μg of ^{125}I-radiolabeled protein (33.3 μg/animal, with a radioactive load of 0.3–1 × 10^7 cpm). The pharmacokinetic parameters were determined using a noncompartmental model with the PKCALC program.

[d] SDs are based on at least three experiments.

mined by SDS-PAGE under nonreducing conditions (18). The F(ab′)$_2$ fragments and IgGs migrated with molecular masses of ~100 and ~150 kDa, respectively. Gel scans showed that the F(ab′)$_2$ preparations of the HER-50, HER-66, and HER-70 mixture as well as Herceptin were >95% pure. No intact IgG could be detected.

As shown in Table 1, when BT474 cells were stained with either the FITC-labeled IgG or F(ab′)$_2$ fragments of the anti-Her-2 MAbs or the IgG or F(ab′)$_2$ fragment of Herceptin, 80–90% of the cells stained positively. Using both the F(ab′)$_2$ fragments and the IgG, HER-70 demonstrated an antigenic density (mean fluorescence intensity) 3× lower than the individual IgG or F(ab′)$_2$ fragments of the anti-Her-2 MAbs or the IgG or F(ab′)$_2$ fragment of Herceptin. The relative binding affinities of all anti-Her-2 constructs were determined by plotting the percentage of positive cells *versus* the MAb concentration and calculating the concentration required to reach 50% saturation. HER-50 and Herceptin showed the highest relative binding affinities on BT474 cells for both the F(ab′)$_2$ fragments and the IgG. HER-70 had the lowest relative binding affinity, both when the F(ab′)$_2$ fragments or the IgG was tested and when HER-66 was intermediary for both the F(ab′)$_2$ fragments and the IgG. The pharmacokinetic parameters for the IgG and F(ab′)$_2$ fragments of one representative MAb, HER-50, were determined using the PKCALC program (21), and the data were calculated using a noncompartmental model. The results indicated that the half-life of the IgG is 5-fold longer than that of its corresponding F(ab′)$_2$ fragment. This correlates with the reported half-life values of 120–200 h for IgG and 12–57 h for F(ab′)$_2$ fragments (27–29).

The Anti-Her-2 IgGs Mediate ADCC and CDC, Whereas Their F(ab′)$_2$ Fragments Do Not. It has been documented that effective antitumor activity of single MAbs requires recognition of tumor cells coupled with the activation of host effector cells via FcRs (17, 30–37). We considered the possibility that this would not be the case when antigrowth activity was optimized using mixtures of MAbs against different epitopes. We therefore compared the effector functions of the mixture of anti-Her-2 F(ab′)$_2$ fragments with those of the mixture of IgGs. As shown in Fig. 2*A* and predicted, in the presence of murine effector cells, the mixture of F(ab′)$_2$ fragments induced <1.5% lysis of target cells at an E:T ratio of 100:1. The IgG mixture induced potent dose-dependent cell death, reaching 70% lysis at an E:T ratio of 100:1. In the presence of human peripheral blood mononuclear cells, at an E:T ratio of 100:1, the F(ab′)$_2$ fragments of Herceptin did not mediate cell lysis, whereas Herceptin mediated 50% cell lysis (data not shown). IgG and F(ab′)$_2$ fragments of the isotype-matched irrelevant control anti-CD25 MAb RFT5 did not induce lysis, whereas NaN$_3$ induced significant lysis. As shown in Fig. 2*B*, the mixture of F(ab′)$_2$ fragments did not fix complement and induced <2.5% cell lysis at the highest dilution (1:10) of mouse serum. The IgG mixture fixed complement, resulting in dose-dependent lysis of the target cells, reaching 45% lysis at a dilution of 1:10 mouse serum. At the same dilution of human serum, Herceptin induced similar lysis (40%), but as predicted, the F(ab′)$_2$ fragments of Herceptin IgG did not (data not shown). The isotype-matched negative controls did not mediate either ADCC or CDC. These results indicate that the F(ab′)$_2$ fragments had no measurable effector function *in vitro*.

Both the IgG and F(ab′)$_2$ Fragments of anti-Her-2 MAbs Inhibit VEGF Secretion by Tumor Cells *in Vitro*. Another proposed mechanism for the antitumor activity of anti-Her-2 MAbs is their anti-angiogenic effect. Hence, anti-Her-2 can down-regulate the production of one or more proangiogenic factors produced by tumor cells (11). We have previously documented the presence of newly formed platelet/endothelial cell adhesion molecule 1 (CD31$^+$) blood vessels in BT474 s.c. breast tumor nodules, suggesting that angiogenesis is involved in their growth (18). We have also shown that anti-Her-2 MAbs
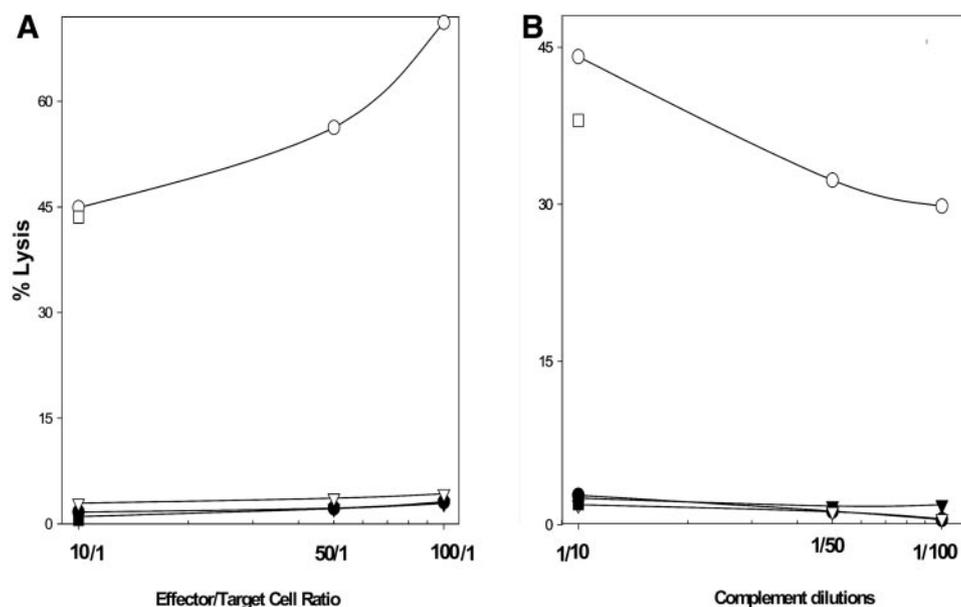
Fig. 2 F(ab′)$_2$ fragments of anti-Her-2 monoclonal antibodies do not induce antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) against BT474 cells *in vitro*. *A* (ADCC), flow cytometric analysis was performed on the 5-(and-6)-carboxyfluorescein diacetate, succinimidylester-stained effector cells (mouse spleen cells) using propidium iodide to stain BT474 cells treated with a mixture of F(ab′)$_2$ fragments (1 μg/10^5 cells) from HER-50, HER-66, and HER-70 (●); the same molar amount of a mixture IgG1s (○); or F(ab′)$_2$ (▼) and IgG (▽) of the isotype-matched control at the same molar concentrations. Negative control BT474 cells were left untreated (■), and positive control cells were treated with 10 mg/ml NaN$_3$ (□). The E:T ratios were 10:1, 50:1, and 100:1. *B* (CDC), target BT474 cells were treated with F(ab′)$_2$ fragments (1 μg/10^5 cells) or the same molar amount of IgGs and mixed with 1:10-, 1:50-, and 1:100-fold dilutions of mouse serum as a source of complement for the CDC assays. Mixture of F(ab′)$_2$ fragments from HER-50, HER-66, and HER-70, ●; mixture of HER-50, HER-66, and HER-70 IgG1s, ○; or F(ab′)$_2$ (▼) and IgG (▽) of the isotype-matched control at the same molar concentrations. Negative control BT474 cells were left untreated (■), and positive control cells were treated with 10 mg/ml NaN$_3$ (□). Both panels depict one representative experiment of three experiments performed. The difference between the percentage of lysis by the mixtures of F(ab′)$_2$ *versus* IgGs is statistically significant ($P = 0.082$). Relative to the isotype-matched control, the effect of the IgG mixture treatment is statistically significant ($P = 2.12 \times 10^{-3}$). The F(ab′)$_2$ mixture is not statistically significant from its isotype-matched control ($P = 0.67$).

can inhibit VEGF production by BT474 cells *in vitro* (18). As shown in Fig. 3, untreated BT474 cells secreted approximately 80 ng VEGF/ml/5 × 10^5 cells over a 72-h period, and treatment with NaN$_3$ inhibited VEGF secretion almost completely. At a concentration of 10^{-7} M, both the mixture of the IgGs and their F(ab′)$_2$ fragments almost completely inhibited VEGF production. The mixture of F(ab′)$_2$ fragments inhibited VEGF production 1.5–3-fold more effectively than each of the individual F(ab′)$_2$ fragments alone and 1.5-fold more effectively than the F(ab′)$_2$ fragments of Herceptin (data not shown). Both as an IgG and as a F(ab′)$_2$, Herceptin induced appreciable but not complete inhibition of VEGF secretion (1.3 and 1.8 ng VEGF/ml/ 5 × 10^5 cells, respectively; data not shown). The IgG and F(ab′)$_2$ fragments of the isotype-matched MAb, RFT5, had no effect at the same concentrations. These results indicate that the mixture of F(ab′)$_2$ fragments of the three anti-Her-2 MAbs is as effective as IgG in blocking the secretion of VEGF.

The *in Vitro* Growth Inhibition and Apoptotic Effect of the Anti-Her-2 IgGs and F(ab′)$_2$ Fragments on BT474 Cells. We next determined whether the anti-Her-2 constructs (IgGs and F(ab′)$_2$ fragments) would inhibit [^{3}H]thymidine incorporation in BT474 cells. The MAbs were incubated for 72 h with BT474 cells, and the levels of [^{3}H]thymidine incorporation were measured and compared with those of untreated cells to determine the IC$_{50}$ values. As shown in Fig. 4, the IC$_{50}$ values of the

mixtures of either IgG or its F(ab′)$_2$ fragments were similar; whereas the IgG and F(ab′)$_2$ fragments of the isotype-matched irrelevant control RFT5 had no effect, NaN$_3$ induced potent killing of the BT474 cells *in vitro*. Table 2 summarizes the mean ± SD of three experiments for both the anti-Her-2 F(ab′)$_2$ fragments and IgGs. When comparing the F(ab′)$_2$ fragments with their corresponding IgGs, the IC$_{50}$ values are similar. The IC$_{50}$ of the F(ab′)$_2$ fragment mixture was 3–26-fold less potent than any one of the three individual F(ab′)$_2$ fragments alone or Herceptin (data not shown). The IC$_{50}$ of the IgG mixture was 3–136-fold less potent (than any one of the three IgGs alone or Herceptin (data not shown).

We also determined whether the mixture of the IgG *versus* F(ab′)$_2$ fragments would induce similar amounts of apoptosis of BT474 cells. Table 2 shows the mean ± SD of three experiments. As measured by annexin-V staining, the mixture of F(ab′)$_2$ fragments induced the most apoptosis, and it was 8–15% higher than that induced by the individual F(ab′)$_2$ fragments or the F(ab′)$_2$ fragments of Herceptin (data not shown). When comparing the IgG mixture with the individual IgGs, the mixture induced slightly more apoptosis, and it was approximately 8–16% higher than that induced by the individual IgGs (data not shown). Herceptin induced 8% more apoptosis than the mixture of individual IgGs (data not shown). The mixture of IgGs and F(ab′)$_2$ fragments induced comparable amounts of
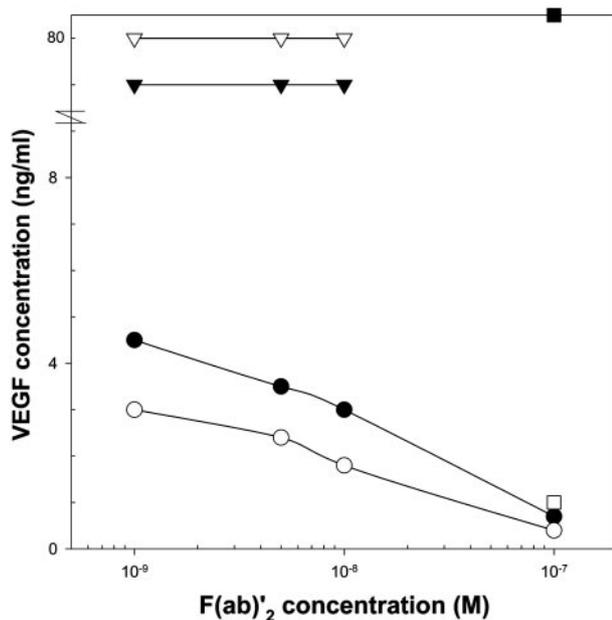
Fig. 3 F(ab')$_2$ fragments of HER-50, HER-66, and HER-70 inhibit the secretion of vascular endothelial growth factor (VEGF) by BT474 cells *in vitro*. Cell supernatants were harvested from 5×10^5 BT474 cells treated with various concentrations of a mixture of F(ab')$_2$ fragments (●) or IgGs at the same molar concentrations (○) and the F(ab')$_2$ (▼) and IgG (▽) of the isotype-matched control at the same molar concentrations. VEGF levels were measured using a competitive ELISA kit. This assay can detect 0.195–200 ng/ml VEGF. Untreated BT474 cells secrete approximately 80 ng VEGF/ml/5×10^5 cells over 72 h (■), and cells treated with 10 mg/ml NaN$_3$ inhibited VEGF secretion (□). The difference between the effect of mixtures of the F(ab')$_2$ *versus* IgGs is not statistically significant ($P = 0.87$). Relative to their isotype-matched controls, the effect of IgG1 mixture and F(ab')$_2$ mixture is statistically significant ($P < 6.95 \times 10^{-4}$. This is a representative experiment of three such experiments performed.

apoptosis. Cell death, as determined by trypan blue exclusion and PI staining, was similar for the mixture of IgGs and F(ab')$_2$ fragments. The mixture of F(ab')$_2$ fragments induced 19–20% more cell death than the individual F(ab')$_2$ fragments (data not shown). The IgG mixture induced 22–28% more cell death than the individual IgGs (data not shown). Levels of apoptosis and necrosis in the untreated controls were negligible. Sodium azide induced significant apoptosis and necrosis. When adding the percentage of apoptotic cells (annexin-V$^+$) to the percentage of necrotic cells (PI$^+$), we observed similar cell death induced by the mixture of IgGs *versus* their F(ab')$_2$ fragments, and this was $1.4\times$ higher than that induced by Herceptin and $2.1\times$ higher than that induced by the F(ab')$_2$ fragments of Herceptin. Herceptin was 1.6-fold more effective in killing cells than its F(ab')$_2$ fragments. These results suggest that the IgG and F(ab')$_2$ fragments of the MAbs have similar activities with regard to antiproliferative and proapoptotic effects.

The Anti-Her-2 F(ab')$_2$ Fragments Have Modest Therapeutic Activity in SCID Mice with s.c. BT474 Tumors. The interactions between the Fc portion of Herceptin and immune effector cells are very important for therapeutic activity (30–32). Both murine MAbs and the humanized, clinically

effective Herceptin engage FcγRIII as well as FcγRIIb (32). To further address the contribution of Fc-mediated effector functions of the mixture of our murine anti-Her-2 MAbs, we used the *in vivo* model described previously (19). In this model, there is 100% tumor take and consistent s.c. tumor growth in preirradiated male SCID mice.

Fig. 5 compares the biodistribution of ^{125}I-HER-50 both as an IgG and a F(ab')$_2$ fragment after i.p. injections of 33.3 μg (4.8×10^8 cpm)/mouse with s.c. tumors. The ^{125}I-HER-50 IgG showed moderate splenic accumulation and negligible localization in kidneys, liver, lungs, and heart. The F(ab')$_2$ fragments localized up to 4–5-fold less effectively than the IgG in s.c. breast tumor nodules, kidneys, liver, lungs, and heart. The splenic accumulation of F(ab')$_2$ fragments commenced after 2 days and was half that of the IgG. The IgG persisted at the tumor site for at least 7 days, whereas the F(ab')$_2$ fragments were cleared after 3 days. In serum, the IgG persisted for 5 days, whereas the F(ab')$_2$ fragments were cleared $4\times$ more rapidly.

The dose used for *in vivo* therapy was adjusted based on the $t_{1/2}$ achieved by injecting ^{125}I-IgG or F(ab')$_2$ fragments of the MAbs. Because of the differences in the $t_{1/2}$ values of IgG *versus* F(ab')$_2$ fragments of the MAbs, 5-fold larger amounts of the F(ab')$_2$ fragments were administered. Therefore, SCID mice with s.c. BT474 tumors measuring 200 mm^3 were treated with
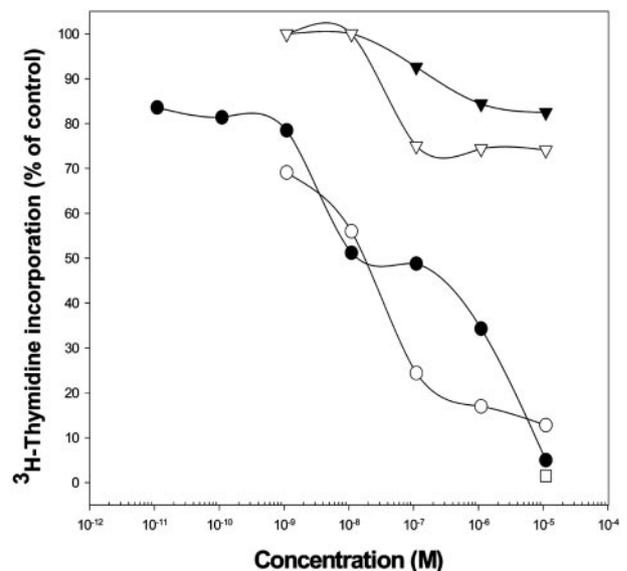

Fig. 4 BT474 cells are killed *in vitro* by both the mixture of IgGs and their F(ab')$_2$ fragments. BT474 cells were treated with varying concentrations of the mixture of F(ab')$_2$ fragments (●), IgGs (○) at the same molar concentrations, and F(ab')$_2$ (▼) and IgG (▽) of the isotype-matched control at the same molar concentrations. Untreated BT474 cells were used as a negative control, and positive control cells were treated with 10 mg/ml NaN$_3$ (□) for 72 h and then pulsed with [^{3}H]thymidine for 6 h. Growth inhibition, expressed as the IC$_{50}$, was calculated by comparing [^{3}H]thymidine incorporation in treated *versus* untreated cells. The difference between the IC$_{50}$ values of the F(ab')$_2$ fragment mixture and the monoclonal antibody mixture is not statistically significant ($P = 0.3$). Relative to the isotype-matched control, the IC$_{50}$ values of F(ab')$_2$ mixture and IgG mixture are statistically significant ($P < 0.02$). This is one representative experiment of three such experiments performed.

Table 2 A mixture of three anti-Her-2 F(ab′)₂ fragments induces similar growth inhibition and apoptosis as the mixture of three anti-Her-2 IgGs *in vitro*

Antibody	$IC_{50} \times 10^{-8}$ (M)[a]	% Annexin-V⁺ cells[b]	% PI⁺ cells[b,c]	% Total dead cells
F(ab′)₂				
Mixture	0.43 ± 0.32[d]	28.0 ± 0.03	31.6 ± 14.5	59.6 ± 7.3
Herceptin	1.3 ± 0.0	15.9 ± 2.9	12.1 ± 3.1	28 ± 3.0
RFT5 (control)	582.5 ± 428.3	2.3 ± 1.6	1.8 ± 1.7	4.1 ± 1.7
IgG1				
Mixture	0.1 ± 0.04	28.4 ± 0.7	38.2 ± 5.9	66.6 ± 3.3
Herceptin	0.3 ± 0.1	36.4 ± 2.2	10.0 ± 8.5	46.4 ± 4.3
RFT5 (control)	233.8 ± 167.8	0.7 ± 0.2	0.7 ± 0.3	1.4 ± 0.3
Other				
None	NA	0.6 ± 0.2	0.64 ± 0.12	1.24 ± 0.2
NaN₃[e]	0.004 ± 0.003	43.8 ± 9.0	49.2 ± 11.9	93 ± 5.3

[a] Cells (2.5×10^5 cells/ml) were incubated for 72 h at 37°C with different concentrations of anti-Her-2 monoclonal antibodies or F(ab′)₂s and then pulsed with [³H]thymidine for 6 h. IC_{50} values represent the concentration of monoclonal antibodies required to kill 50% of cells. The difference between the IC_{50} in each treatment group *versus* untreated control is statistically significant, with $Ps < 0.01$.

[b] Cells (2.5×10^5 cells/ml) were incubated for 4 h at 37°C with 300 μg/ml anti-Her-2 monoclonal antibodies or F(ab′)₂s and then stained with annexin-V FITC plus propidium iodide (50 μg/ml) and analyzed by FACScan. The percentage of total dead cells is the sum of the percentage of annexin-V-FITC-positive plus propidium iodide-positive cells. The difference between the percentage of annexin-V-positive cells in each treatment group *versus* untreated control is statistically significant, with P values < 0.04. The difference between the percentage of propidium iodide-positive cells in each treatment group *versus* untreated control is statistically significant, with P values < 0.01.

[c] PI, propidium iodide; NA, not applicable (because the untreated control values were taken as 100%).

[d] SD are based on at least three experiments.

[e] Positive control.

one dose of 75 μg/g mouse of the mixture of anti-Her-2 F(ab′)₂ fragments or Herceptin F(ab′)₂, 10 weeks after tumor cell inoculation. The IgG mixture or Herceptin was administered as one i.p. injection of 15 μg/g mouse. RFT5 F(ab′)₂ fragments (75 μg/g mouse) were injected i.p. in one group of control mice.

Individual variations of tumor nodules were used to calculate in the mean values ± SD for the five mice in each treatment group.

As shown in Fig. 6, tumors in mice treated with the both the mixture of anti-Her-2 MAbs and Herceptin regressed commencing immediately after the treatment, and after 2 weeks,
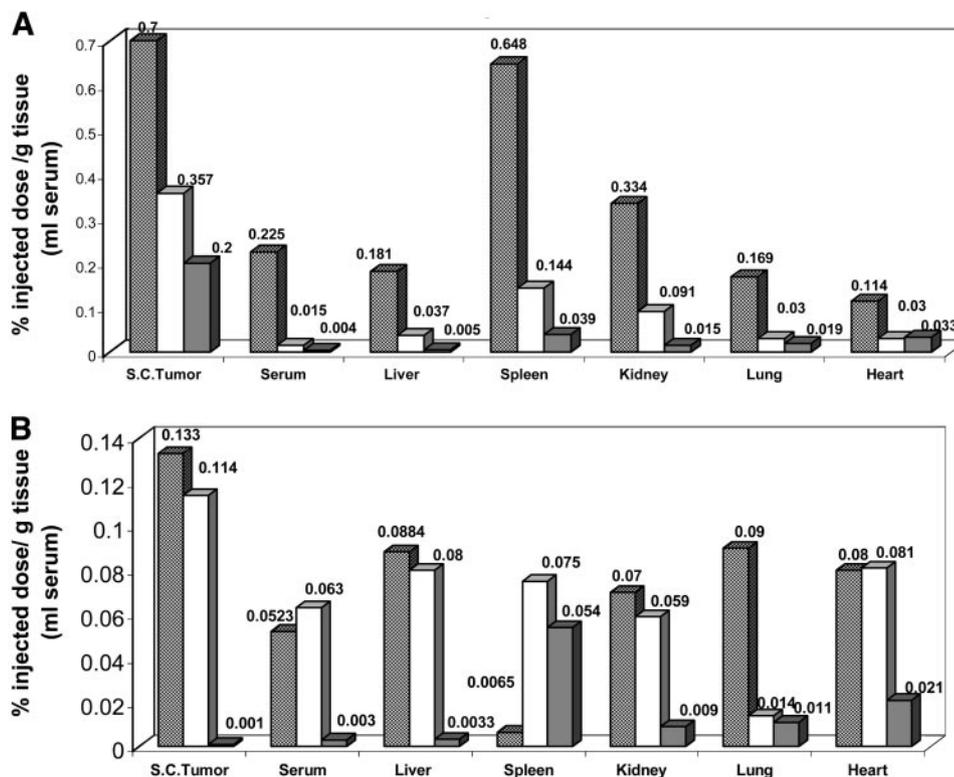
Fig. 5 Biodistribution of ¹²⁵I-HER-50 IgG1 or ¹²⁵I-HER-50 F(ab′)₂ in tumor-bearing mice. After i.p. injection of a dose of 33.3 μg/animal (4.8×10^8 cpm) of radiolabeled HER-50 IgG (*A*) or its F(ab′)₂ fragment (*B*), levels of radioactivity were measured at 24 (*hatched box*), 48 (*white box*), and 72 h (*gray box*) using a gamma counter. The results are expressed relative to the injected dose and organ weight or ml of serum (absolute percentage of initial injected dose/g tissue or ml of serum). This is one representative experiment of three such experiments performed.
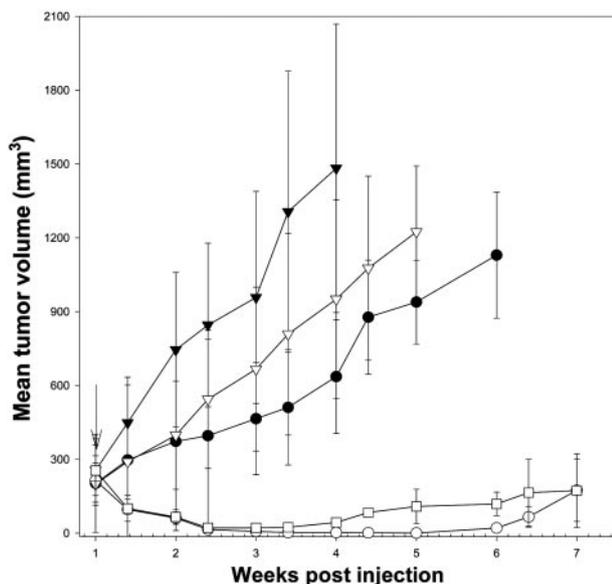
Fig. 6 The mixture of F(ab′)$_2$ fragments is less effective than the mixture of anti-Her-2 monoclonal antibodies *in vivo*. Because of the differences in half-lives between the IgGs and the F(ab′)$_2$ values *in vivo*, 5-fold greater amounts of the F(ab′)$_2$ monoclonal antibodies were administered. Groups of five preirradiated severe combined immunodeficient mice with 200-mm^3 s.c. BT474 breast tumors received i.p. injection with one dose of 1500 μg (75 μg/g mouse) of a mixture of F(ab′)$_2$ fragments (●), 300 μg (15 μg/g mouse) of a mixture of the three IgGs (○), 75 μg/g mouse of F(ab′)$_2$ fragments of the isotype-matched control (▼), 75 μg/g mouse of F(ab′)$_2$ fragments of Herceptin (▽), and 15 μg/g mouse Herceptin IgG (□). The mean tumor volume (mm^3) ± SD for the five mice in each group was plotted. The difference between the effects of the mixture of our murine IgGs and Herceptin IgG *versus* the control are significant ($Ps < 1.3 \times 10^{-5}$). The difference between the effects of the mixture of F(ab′)$_2$ and Herceptin F(ab′)$_2$ *versus* the control is not significant ($Ps < 0.1$). The differences between the effects of the mixture of F(ab′)$_2$ and IgG and between Herceptin F(ab′)$_2$ and Herceptin IgG are significant ($Ps < 7.6 \times 10^{-4}$). Animals were euthanized when tumor burdens exceeded 1500 mm^3 or 10% of total body weight.

tumor nodules became nonpalpable for 3 weeks. The mixture of F(ab′)$_2$ fragments of anti-Her-2 MAbs or Herceptin induced 50% and 49% reductions in tumor volumes, followed by relapse within a week. The group treated with F(ab′)$_2$ fragments of the isotype-matched RFT5 control showed continuous progressive growth. All mice survived for the same length of time, but all were sacrificed when tumor burdens exceeded 1500 mm^2 in volume or 10% of total body weight. These experiments demonstrate that FcR-dependent mechanisms are largely responsible for the effect of our mixture of three anti-Her-2 MAbs tested here. Thus, F(ab′)$_2$ fragments of the same anti-Her-2 MAb mixture as well as Herceptin had only modest antitumor activity. The growth inhibition achieved by one dose of the IgG mixture was more rapid, potent, and long-lived than that achieved by a 5-fold higher dose of the F(ab′)$_2$ mixture. It is possible but unlikely that small, undetectable amounts of IgG (<5% or a maximum of 75 μg) contaminating the F(ab′)$_2$ mixture accounted for the observed antitumor activity, although we could detect no ADCC or CDC activity *in vitro* even at an E:T ratio of 100:1 or a 1:10 dilution of complement-containing serum.

DISCUSSION

We have demonstrated previously (18) that a mixture of three anti-Her-2 MAbs, which bind to different epitopes on the extracellular domain of Her-2, had more potent antitumor activity *in vitro* and *in vivo* than the individual MAbs. The mechanisms underlying this improved activity could include increased blocking of signaling pathways, more effective activation of apoptosis, greater inhibition of angiogenesis, and improved effector cell-mediated cytotoxicity. Indeed, all these improved activities could be demonstrated *in vitro*. However, their contributions to the improved *in vivo* efficacy of our MAbs are unclear. In this regard, over the past several years, it has been shown that much, if not all, of the *in vivo* activity of several therapeutic antibodies (including Herceptin) is due to Fc-mediated effector function (32, 38). Thus, using FcR knockout mice or immunoglobulin molecules engineered not to bind to FcRs has resulted in the loss of antitumor activity. Although these results are compelling, they apply to single MAbs and not to mixtures of MAbs, which signal much more effectively. For this reason, we carried out this study to determine whether this increased signaling would overcome the need for Fc-mediated effector function. To this end, we compared the antitumor activity of our three anti-Her-2 IgGs (HER-50, HER-66, and HER-70) with their F(ab′)$_2$ fragments. Herceptin, the fully humanized version of the anti-Her-2 MAb 4D5, was used as a reference MAb.

The major findings to emerge from this study are as follows. (*a*) IgG and F(ab′)$_2$ fragments of the MAb mixture had almost identical antiproliferative and proapoptotic activity *in vitro*. As shown previously for their IgGs (19), the antitumor activity of a mixture of the three F(ab′)$_2$ fragments was superior to that of the individual MAbs. (*b*) As expected, the F(ab′)$_2$ fragments (which were >95% pure) did not induce ADCC or CDC *in vitro*. (*c*) As compared with the F(ab′)$_2$, the IgG had a longer half-life in the circulation and in the tumor site, with modest accumulation in the spleen. The F(ab′)$_2$ fragments were cleared 4–5 times more rapidly from the circulation and the tumor site, and the uptake in normal organs was negligible. (*d*) In contrast to their similar activities *in vitro*, the mixture of IgGs was much more effective than the mixture of their F(ab′)$_2$ fragments in inducing tumor regressions in SCID mice with breast carcinoma xenografts, even when the latter were administered at 5-fold higher concentrations to compensate for their shorter half-lives. These results clearly show that *in vivo* activity is largely Fc dependent, as suggested previously for Herceptin alone (31). Whereas the F(ab′)$_2$ fragments of the mixture of anti-Her-2 MAbs had some activity *in vivo*, it was modest and short-lived. Furthermore, although we consider it unlikely, we cannot exclude the possibility that this activity was due either completely or in part to trace amounts of contaminating IgGs because 5-fold higher doses of F(ab′)$_2$ fragments as compared with IgG were administered. Hence, our studies confirm and extend the observation that the *in vivo* antitumor activity of anti-Her-2 MAbs, either alone or as a mixture, is largely Fc dependent, despite significant Fc-independent activity *in vitro* (30, 39).

Anti-Her-2 antibodies have several modes of action *in vitro*, and many of these are Fc independent. For example, they

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 the transcription of growth factors (40–42). Anti-Her-2 MAbs can also inhibit angiogenesis by blocking Her-2-mediated signal transduction pathways that up-regulate the expression of proangiogenic factors such as VEGF.

In contrast to these many *in vitro* activities, several studies have suggested that the antitumor activity of MAb therapy *in vivo* is due predominantly to ADCC/CDC (43–46). These activities require the Fc portion of the MAbs and FcγRs on effector cells and the classical pathway of complement activation (7–9, 47). Anti-Her-2 MAbs can kill tumor cells *in vitro* through such immunological mechanisms (44–47), and these effects are dependent on the immunoglobulin isotype of the MAb. It has been proposed that the *in vivo* antitumor activity of MAbs results from the sum of the opposing activation and inhibition of immune cells through FcγRs (30). MAbs against Her-2, such as Herceptin and its murine 4D5 counterpart, engage both FcγRIII and FcγRIIb receptors on immune cells (30). Importantly, deletion of the FcγRIII in mice eliminates virtually all of the *in vivo* antitumor activity of both 4D5 and Herceptin (30). In contrast, the antibodies were more effective in mice lacking the inhibitory FcγRIIb receptor (30, 48).

It is also known that the effectiveness of a MAb *in vivo* depends on whether the Fc portion of the antibody is of the same species as the species being treated (49, 50). The Fc portion of some 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 (49). We have shown previously that Herceptin mediates ADCC/CDC *in vitro* when human immune effector cells and serum are used but that it is very ineffective when murine immune effector cells and sera are used (19). Hence, the activity of Herceptin in mice with human tumor xenografts gives an underestimate of its potency in humans.

In summary, the results presented in this study suggest that *in vitro* antiproliferative and antiangiogenic functions of the F(ab′)$_2$ fragments of the three anti-Her-2 MAbs studied are comparable with their corresponding IgGs. In contrast, as compared with the IgG mixture, the F(ab′)$_2$ mixture has inferior antitumor activity *in vivo*. Because F(ab′)$_2$ fragments cannot induce ADCC or CDC, the *in vivo* antitumor activity of the MAb mixture, like that of Herceptin alone (44, 49), is due predominantly to Fc-dependent activity (30). Taken together with the results of others, our data strongly suggest that it is not desirable to remove the Fc portion of anti-Her-2 therapeutic antibodies, even when they are used as mixtures with superior antigrowth activities.

ACKNOWLEDGMENTS

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A Comparison of the *in Vitro* and *in Vivo* Activities of IgG and F(ab´)$_2$ Fragments of a Mixture of Three Monoclonal Anti-Her-2 Antibodies

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