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Recombinant Anti-Human HER2/neu IgG3-(GM-CSF) Fusion Protein Retains Antigen Specificity and Cytokine Function and Demonstrates Antitumor Activity

Jay S. Dela Cruz, K. Ryan Trinh, Sherie L. Morrison, and Manuel L. Penichet

Anti-HER2/neu therapy of human HER2/neu-expressing malignancies such as breast cancer has shown only partial success in clinical trials. To expand the clinical potential of this approach, we have genetically engineered an anti-HER2/neu IgG3 fusion protein containing GM-CSF. Anti-HER2/neu IgG3-(GM-CSF) expressed in myeloma cells was correctly assembled and secreted. It was able to target HER2/neu-expressing cells and to support growth of a GM-CSF-dependent murine myeloid cell line, FDC-P1. The Ab fusion protein activated J774.2 macrophage cells so that they exhibit an enhanced cytotoxic activity and was comparable to the parental Ab in its ability to effect Ab-dependent cellular cytotoxicity-mediated tumor cell lysis. Pharmacokinetic studies showed that anti-HER2/neu IgG3-(GM-CSF) is stable in the blood. Interestingly, the half-life of anti-HER2/neu IgG3-(GM-CSF) depended on the injected dose with longer in vivo persistence observed at higher doses. Biodistribution studies showed that anti-HER2/neu IgG3-(GM-CSF) is mainly localized in the spleen. In addition, anti-HER2/neu IgG3-(GM-CSF) was able to target the HER2/neu-expressing murine tumor CT26-HER2/neu and enhance the immune response against the targeted Ag HER2/neu. Anti-HER2/neu IgG3-(GM-CSF) is able to enhance both Th1- and Th2-mediated immune responses and treatment with this Ab fusion protein resulted in significant retardation in the growth of s.c. CT26-HER2/neu tumors. Our results suggest that anti-HER2/neu IgG3-(GM-CSF) fusion protein is useful in the treatment of HER2/neu-expressing tumors. The Journal of Immunology, 2000, 165: 5112–5121.

The HER2/neu protooncogene (also known as c-erbB-2) encodes a 185-kDa transmembrane glycoprotein receptor known as HER2/neu or p185HER2 that has partial homology with the epidermal growth factor receptor and shares with that receptor intrinsic tyrosine kinase activity (1–3). It consists of three domains: a cysteine-rich extracellular domain; a transmembrane domain; and a short cytoplasmic domain (1–3). Overexpression of HER2/neu is found in 25–30% of human breast cancer and this overexpression is an independent predictor of both relapse-free and overall survival in breast cancer patients (4–7). Overexpression of HER2/neu also has prognostic significance in patients with ovarian (5), gastric (6), endometrial (9), and salivary gland cancers (10). The increased occurrence of visceral metastasis and micro-metastatic bone marrow disease in patients with HER2/neu overexpression has suggested a role for HER2/neu in metastasis (11, 12).

The elevated levels of the HER2/neu protein in malignancies and the extracellular accessibility of this molecule make it an excellent tumor-associated Ag (TAA)3 for tumor-specific therapeutic agents. In fact, treatment of patients with advanced breast cancer using the anti-HER2/neu Ab, trastuzumab (Herceptin, Genentech, San Francisco, CA), previously known as rhuAb HER2, directed at the extracellular domain of HER2/neu (ECDHER2) (13), can lead to an objective response in some patients with tumors overexpressing the HER2/neu oncoprotein (14, 15). However, only a subset of patients shows an objective response (5 of the 43 (11.6%) (14, 15). Although combination of trastuzumab with chemotherapy enhances its antitumor activity (9 of 37 patients with no complete response (24.3%)) (16), improved therapies are still needed for the treatment of HER2/neu-expressing tumors.

GM-CSF is a cytokine associated with the growth and differentiation of hemopoietic cells. It is also a potent immunostimulator with pleiotropic effects, including the augmentation of Ag presentation in a variety of cells (17–22), increased expression of MHC class II on monocytes and adhesion molecules on granulocytes and monocytes (23–25), and amplification of T cell proliferation (26). In animals, the injection of GM-CSF potentiates the protective effects of an antitumor vaccine by enhancing T cell immunity (26), and vaccination with GM-CSF-transduced cells has been shown to be effective in the treatment of experimental tumors in murine models (27–30).

Studies suggest that for GM-CSF to be effective it must be concentrated in the vicinity of the tumor, where it acts in a paracrine manner. A completed phase I clinical trial showed that vaccination of patients with metastatic melanoma with irradiated autologous melanoma cells engineered to secrete human GM-CSF-stimulated potent antitumor immunity (31). Although the results suggest that

1 This work was supported in part by Grant 3CB-0245 from the University of California at Los Angeles.
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3 Abbreviations used in this paper: TAA, tumor-associated Ag; DNS, N,N-dimethyl-1-aminonaphthalene-5-sulfonyl chloride (dansyl); rmGM-CSF, recombinant murine GM-CSF; ECDHER2, extracellular domain of HER2/neu Ag; AP, alkaline phosphatase; ADCC, Ab-dependent cellular cytotoxicity; %ID/g tissue, percent of injected dose per gram of tissue.
this immunization strategy has potential application in the treatment of minimal residual disease, the ex vivo genetic modification and reintroduction of cells into patients is limited by its patient-specific nature. Additionally, it is technically difficult, time consuming, and expensive to expand primary autologous human tumor cells to the numbers required for vaccination (31–34). Although in vivo gene delivery using viral vectors has been considered, the low transfer efficiency of retroviral vectors and the immunogenicity of adenoviral vectors have limited efficacy (34). Although systemic administration of GM-CSF is an alternative approach, patients in clinical trials receiving high doses of GM-CSF have experienced severe toxic side effects (35) including a reported fatigue (36), and no significant antitumor activity has been achieved. Thus, the challenge of developing an effective approach for achieving high local concentrations of GM-CSF remains.

Ab-(GM-CSF) fusion proteins that recognize TAAIs provide one approach for achieving effective GM-CSF-mediated immune stimulation at the site of the tumor. In the present report, we characterize a novel Ab fusion protein, anti-HER2/neu IgG3-(GM-CSF) containing the variable region of the humanized anti-HER2/neu Ab, trastuzumab (Herceptin, Genentech, San Francisco, CA), and the murine GM-CSF. The properties of anti-HER2/neu IgG3-(GM-CSF) suggest that it may provide an effective alternative for the therapy of HER2/neu-expressing tumors.

Materials and Methods

Cell lines

CT26 is a murine colon adenocarcinoma that was induced in BALB/c mice by intraperitoneal injection of N-nitroso-N-methylurethane (37, 38). It was provided by Dr. Young Chul Sung (Pohang University of Science and Technology, Pohang, Korea). CT26-HER2/neu was developed in our laboratory by transduction of CT26 cells with the DNA-encoding human HER2/neu (39). We previously showed that this cell line is able to grow in immunocompetent mice while maintaining the expression of human HER2/neu on its surface (39).

J774.2, a murine macrophage cell line was obtained from Dr. Mathew Scharff (Albert Einstein College of Medicine, Bronx, NY). The P3X63Ag8.653 mouse nonproducing myeloma was purchased from the American Type Culture Collection (ATCC, Manassas, VA). These four cell lines (CT26, CT26-HER2/neu, J774.2, and P3X63Ag8.653) were cultured in IMDM supplemented with 5% bovine calf serum, L-glutamine, penicillin, and streptomycin. The GM-CSF-dependent murine myeloid cell line, FDC-P1, purchased from the ATCC, was cultured in IMDM supplemented with 10% FBS containing 25% WEHI-3-conditioned medium, L-glutamine, penicillin, and streptomycin. All cells were incubated at 37°C in the presence of 5% CO2.

Mice

Female BALB/c mice 6–8 wk of age obtained from Taconic Farms (Germantown, NY) were used. All experiments were performed according to published procedures (40). Animals were housed in a facility using autoclaved polycarbonate cages containing wood shaving bedding. The animals received food and water ad libitum. Artificial light was provided under a 12/12-h light/dark cycle. The temperature of the facility was 20°C with 10–15 air exchanges per hour.

Vector construction, transfection, and initial characterization of anti-human HER2/neu IgG3-C3-Gm3-(GM-CSF)

The DNA encoding the variable light (Vλ) and heavy (VH) chain domains of the humanized Ab human4DS-8 (13) (15) or rhuMAB HER2 (14, 16) (generously provided by Paul Carter, Genentech) had previously been cloned into mammalian expression vectors for human λ light chain and IgG3 heavy chain, respectively (41). The mature form of murine GM-CSF was amplified from the plasmid pCEP4/GM-CSF generously provided by Dr. Mi-Hua Tao (Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan) by PCR using the sense primer 5′-CCCTCCCCAGGGACCCACCCAC CGGCTACACCC-3′ and the antisense primer 5′-CGGAATTCGTTTAAC CTTTTGGACTGTTTTTGCATTC-3′.

The PCR product was digested with NruI/EcoRI and cloned in the vector pAT3462 (previously developed in our laboratory) digested with SphI/EcoRI, yielding the vector pAT1791 (Fig. 1). The plasmids pAT6611, pAH4874 (both previously developed in our laboratory), and pAT1791 were digested with EcoRV/NsiI, EcoRV/BamHI, and NsiI/BamHI, respectively. The fragments containing the DNA encoding for anti-HER2/neu VH and γ3 constant regions (from pAT6611), the expression vector backbone (from pAH4874), and GM-CSF (from pAT1791) were purified using a Qiagen (Chatsworth, CA) Gel Extraction Kit after electrophoresis in an 0.8% agarose gel. The three fragments were ligated, yielding the anti-human HER2/neu IgG3-C3-Gm3-(GM-CSF) heavy chain expression vector pAH1792. A cell line that produces high levels of anti-human HER2/neu κ light chain, TAOL 5.2.3, was first obtained by transfecting P3X63Ag8.653 by electroporation with the mammalian expression vector for human anti-human HER2/neu κ (Fig. 1) and selecting resistant mycoplasmic acid-stable transfectants. These were screened for L-chain secreting and ELISA (42). The heavy chain was expressed as reference pAH1792 clone 1.2 to electroporate the light chain producer TAOL 5.2.3 (Fig. 1). Stable transfectants were selected with 5 mM histidinol (Sigma, St. Louis, MO) and screened by ELISA for the secretion of heavy chain (42). Transfectants were biosynthetically labeled with [35S]methionine (ICN, Irvine, CA), and the fusion protein was immunoprecipitated using rabbit anti-human IgG and polyclonal suspension of staphylococcal protein A (IgG-Sepharose, Sigma, St. Louis, MO) and analyzed by SDS-PAGE with or without reduction by β-ME. The fusion protein was purified from culture supernatants using protein A immobilized on Sepharose 4B fast flow (Sigma). Protein concentrations were determined by bichinchoninic acid-based protein assay (BCA Protein Assay; Pierce, Rockford, IL) and ELISA. Purity and integrity were assessed by Coomassie blue staining of proteins separated by SDS-PAGE. The potential presence of aggregates in the purified protein was studied by fast protein liquid chromatography (Superose 6, Amersham Pharmacia Biotech, Piscataway, NJ) in filtered and degassed PBS + 0.02% sodium azide.

Ag binding

CT26 or CT26-HER2/neu (106) cells were incubated with 1 μg anti-HER2/neu IgG3-(GM-CSF) in 0.1 ml PBS plus 2% of bovine calf serum for 2 h at 4°C. Flagging the anti-HER2/neu IgG3-(GM-CSF) (41) and selecting resistant mycobacterial protein A (IgG-Sepharose, Sigma, St. Louis, MO) and analyzed by SDS-PAGE with or without reduction by β-ME. The fusion protein was purified from culture supernatants using protein A immobilized on Sepharose 4B fast flow (Sigma). Protein concentrations were determined by bichinchoninic acid-based protein assay (BCA Protein Assay; Pierce, Rockford, IL) and ELISA. Purity and integrity were assessed by Coomassie blue staining of proteins separated by SDS-PAGE. The potential presence of aggregates in the purified protein was studied by fast protein liquid chromatography (Superose 6, Amersham Pharmacia Biotech, Piscataway, NJ) in filtered and degassed PBS + 0.02% sodium azide.

Prefusion assay

The GM-CSF-dependent murine myeloid cell line FDC-P1 was used to study the bioactivity of anti-HER2/neu IgG3-(GM-CSF), rmGM-CSF from Escherichia coli with ED₅₀ ≤ 0.2 ng/ml (Chemicon, Temecula, CA) reconstituted using deionized water following the manufacturer’s recommendations and stored at −20°C. Serial dilutions of equivalent molar concentrations of rmGM-CSF and anti-HER2/neu IgG3-(GM-CSF) were made in RPMI 1640 + 10% FBS in a volume of 0.1 ml PBS plus 2% bovine calf serum. Cells were washed and incubated for 2 h at 4°C with 0.5 μg biotinylated goat anti-human IgG (PherMingen, San Diego, CA) in a volume of 0.1 ml of PBS plus 2% bovine calf serum. Cells were washed and incubated for 30 min with 0.03 μg PE-labeled streptavidin (PherMingen) in a volume of 0.1 ml PBS plus 2% bovine calf serum. Analysis was performed by flow cytometry with a FACScan (Becton Dickinson, Mountain View, CA) equipped with a blue laser excitation of 15 mW at 488 nm.

Macrophage-mediated cytotoxicity

Macrophage-mediated cytotoxicity was performed according to the methods of Duerst and Werberig (43) using the DNA fragmentation assay of Matzinger (44) with modifications. Briefly, the target cells CT26-HER2/neu were labeled with [3H]thymidine (ICN) at 5 μCi/ml (sp act 6.7 Ci/mmol) in IMDM supplemented with 5% bovine calf serum for 24 h at 37°C. Labeled target cells were washed with medium and incubated with rmGM-CSF at the phenotypic effect. In the presence of 5 μg/ml anti-HER2/neu IgG3, the molar equivalent amount of anti-HER2/neu IgG3-(GM-CSF) or no Ab for 24 h at 37°C. Alternatively, J774.2 cells were incubated with 6.7 × 10⁻² μg/ml anti-HER2/neu IgG3-(GM-CSF) (equivalent to 50
U/ml GM-CSF portion of anti-HER2/neu IgG3-(GM-CSF), with anti-HER2/neu IgG3 at a concentration equivalent to the Ab portion of anti-HER2/neu IgG3-(GM-CSF) (5.68 × 10^{-7} μg/ml), or with no additions in IMDM supplemented with 5% bovine calf serum for 24 h at 37°C. After incubation, the J774.2 cells were washed with medium and then transferred into a 96-well round-bottom tissue culture plate (Costar) containing 1 × 10^5 [3H]thymidine-labeled CT26-HER2/neu per well (E:T 10). All incubations were conducted for 24 h in a final volume of 200 μl/well using IMDM supplemented with 5% bovine calf serum and 50 μM cold thymidine. The presence of 50 μM cold thymidine blocks the incorporation of released [H]thymidine by the J774.2 effector cells (43). The cells were harvested and passed through a glass-fiber filter (Wallac Oy, Turku, Finland) using a Micro Cell Harvester (Skatron, Lier, Norway). Labeled DNA from intact target cells was captured by the filters. The radioactivity was measured with a 1205 Betaplate Liquid Scintillation Counter (Wallac Oy, Turku, Finland). The percent cytotoxicity mediated by J774.2 macrophage cells was calculated as 100 × (cpm test/cpm control) (44). Data are presented as %ID/g tumor.

Biodistribution

Groups of 4 mice were sacrificed 4 or 16 h after the i.v. injection of 1 μCi 125I-labeled anti-HER2/neu IgG3-(GM-CSF). At various intervals after injection of 125I-labeled anti-HER2/neu IgG3-(GM-CSF), residual radioactivity was measured using a mouse whole body counter (Wn. B. Johnson, Montville, NJ). Blood samples were obtained from the tail vein of mice 2, 4, and 12 h after injection. Serum was separated from clotted blood and stored at −20°C until assayed by SDS-PAGE to confirm the integrity of the protein. Values are corrected for the radioactivity in blood in each tissue using the values of blood volume corresponding to each organ (45).

Tumor targeting

Anti-HER2/neu IgG3-(GM-CSF) was iodinated to ~2 μCi/μg with 125I using Iodo-Beads (Pierce) according to manufacturer’s protocol. Mice were injected i.v. via the lateral tail vein with 1 μCi 125I-labeled proteins alone or mixed with 20 μg cold anti-HER2/neu IgG3-(GM-CSF). At various intervals after injection of 125I-labeled anti-HER2/neu IgG3-(GM-CSF), residual radioactivity was measured using a gamma counter (Gamma 5500, Beckman Coulter, Fullerton, CA). Data are presented as %ID/g tissue. Statistical analysis of the biodistribution was conducted using the Mann-Whitney rank test, and the statistical analysis of the DNA fragmentation assay and the antitumor experiments was done using a two-tailed Student t test. For all cases, results were regarded significant if p values were ≤0.05.

Results

Construction, expression, and initial in vitro characterization of anti-HER2/neu IgG3-C mu 3-(GM-CSF)

The strategy for the construction and expression of anti-HER2/neu IgG3-C mu 3-(GM-CSF) is illustrated in Fig. 1. Clones expressing anti-HER2/neu IgG3-C mu 3-(GM-CSF) were identified by ELISA and biosynthetically labeled by growth in the presence of [35S]methionine. Labeled secreted protein was immunoprecipitated using rabbit anti-human IgG and analyzed by SDS-PAGE under reducing and nonreducing conditions. The anti-HER2/neu IgG3-C mu 3-(GM-CSF) was correctly assembled and secreted and exhibits the expected m.w. (data not shown). These results were confirmed by SDS-PAGE of purified proteins. In the absence of reducing agents anti-HER2/neu IgG3 migrates with an apparent molecular mass of 170 kDa whereas anti-HER2/neu IgG3-(GM-CSF) is ~200 kDa, the size expected for a complete IgG3 with 2 molecules of GM-CSF attached (Fig. 2). After treatment with the reducing agent, light chains migrating with an apparent molecular mass of ~25 kDa are seen for both proteins. However, the anti-HER2/neu IgG3 has a heavy chain with an apparent molecular mass of ~60 kDa, whereas anti-HER2/neu IgG3-(GM-CSF) has a heavy chain with an apparent molecular mass of ~75 kDa (Fig. 2B) as expected. Thus, proteins of the expected molecular mass are produced and fusion of murine GM-CSF to the carboxyl terminus of the heavy chain of anti-HER2/neu IgG3 does not appear to alter the assembly and secretion of the H-s subunit of the Ab fusion protein. Analysis of anti-HER2/neu IgG3 and anti-HER2/neu IgG3-(GM-CSF) by fast protein liquid chromatography under nondenaturing conditions showed that both proteins eluted as a single peak of the expected m.w. with no evidence of aggregation (data not shown).

Ag binding at the cell surface

The ability of anti-HER2/neu IgG3-(GM-CSF) to bind to the HER2/neu target Ag was examined using flow cytometry. Both anti-HER2/neu IgG3-(GM-CSF) and anti-HER2/neu IgG3 specifically bound to the human HER2/neu expressed on the surface of the murine cell line CT26-HER2/neu (Fig. 3, B and C). The same

Statistical analysis

Statistical analysis of the titration ELISA was conducted using the Mann-Whitney rank test, and the statistical analysis of the DNA fragmentation assay and the antitumor experiments was done using a two-tailed Student t test. For all cases, results were regarded significant if p values were ≤0.05.

U/ml GM-CSF portion of anti-HER2/neu IgG3-(GM-CSF), with anti-HER2/neu IgG3 at a concentration equivalent to the Ab portion of anti-HER2/neu IgG3-(GM-CSF) (5.68 × 10^{-7} μg/ml), or with no additions in IMDM supplemented with 5% bovine calf serum for 24 h at 37°C. After incubation, the J774.2 cells were washed with medium and then transferred into a 96-well round-bottom tissue culture plate (Costar) containing 1 × 10^5 [3H]thymidine-labeled CT26-HER2/neu per well (E:T 10). All incubations were conducted for 24 h in a final volume of 200 μl/well using IMDM supplemented with 5% bovine calf serum and 50 μM cold thymidine. The presence of 50 μM cold thymidine blocks the incorporation of released [H]thymidine by the J774.2 effector cells (43). The cells were harvested and passed through a glass-fiber filter (Wallac Oy, Turku, Finland) using a Micro Cell Harvester (Skatron, Lier, Norway). Labeled DNA from intact target cells was captured by the filters. The radioactivity was measured with a 1205 Betaplate Liquid Scintillation Counter (Wallac Oy, Turku, Finland). The percent cytotoxicity mediated by J774.2 macrophage cells was calculated as 100 × (cpm test/cpm control) (44). Data are presented as %ID/g tumor.

Biodistribution

Groups of 4 mice were sacrificed 4 or 16 h after the i.v. injection of 1 μCi (0.5 μg) 125I-labeled anti-HER2/neu IgG3-(GM-CSF). Various organs and blood were collected and weighed, and radioactivity was measured using a gamma counter (Gamma 5500, Beckman Coulter, Fullerton, CA). Data are presented as % of injected dose per gram of tissue (%ID/g tissue). Values were corrected for the radioactivity in blood in each tissue using the values of blood volume corresponding to each organ (45).

Tumor targeting

Anti-HER2/neu IgG3-(GM-CSF) was iodinated as described above. CT26 and CT26-HER2/neu cells (10^5 in 0.15 ml HBSS (Life Technologies, Grand Island, NY)) were injected separately into the left and right flanks of three mice. Seven days after tumor injection when tumors were ~1.0 cm in diameter, the three mice were injected i.v. via the lateral tail vein with 6 μCi 125I-labeled anti-HER2/neu IgG3-(GM-CSF). Mice were euthanized 12 h after injection of anti-HER2/neu IgG3-(GM-CSF). Tumors and blood were removed and weighed, and radioactivity was measured with a gamma counter. Data are presented as %ID/g tumor.

Immunotherapy

CT26-HER2/neu cells (1 × 10^6 in 0.15 ml HBSS) were injected s.c. into the right flank of syngeneic BALB/c mice. Beginning the next day, mice randomized into groups of eight received serially i.v. injections of 0.25 ml PBS containing 20 μg anti-HER2/neu IgG3-(GM-CSF), the equivalent molar amount of anti-HER2/neu IgG3, or nothing. Tumor growth was monitored and measured with a caliper every 3 days until day 15 at which time mice were euthanized. Blood samples were collected, and serum was separated from clotted blood and stored at −20°C until assayed by ELISA.

Determination of murine anti-human HER2/neu and anti-human IgG3 Abs

Sera from each treatment group were analyzed by ELISA for the presence of Abs to human IgG3 and human HER2/neu using 96-well microtiter plates coated with 50 μl anti-human HER2/neu IgG3 or human ECD1/H2 (at a concentration of 1 μg/ml), respectively. The plates were blocked with 3% BSA in PBS, and dilutions of serum in PBS containing 1% BSA were added to the wells and incubated overnight at 4°C. After a washing with PBS, alkaline phosphatase (AP)-labeled goat anti-mouse IgG (Sigma) was added, and the plates were incubated for 1 h at 37°C. After a washing, p-nitrophenyl phosphate disodium dissolved in diethanolamine buffer (Sigma) was added to the wells for 1 h, and plates were read at 410 nm. Sera from mice of the same age bearing tumors of the parental cell line CT26 was used as a negative control for determining anti-HER2/neu titers. Sera from naive mice of the same age were used as a negative control for determining anti-human IgG3 titers. All ELISAs for comparison of titers between the experimental groups were made simultaneously in duplicate using an internal positive control curve for each plate.

Determination of isotype profile of murine anti-human HER2/neu and anti-human IgG3 Abs

The isotype of the murine anti-human IgG3 and anti-human HER2/neu was determined by ELISA using 96-well microtiter plates prepared as described above. Pooled sera from each treatment group diluted 1:50 in 1% BSA in PBS was added at 50 μl/well in duplicate into the 96-well plates and allowed to stand overnight at 4°C. After the plates were washed with PBS, rat Abs specific for murine IgG2a, IgG2b, IgG3, or κ (PharMingen) diluted in 1% BSA in PBS were added to each well and incubated 2 h at room temperature. After washing with PBS, alkaline phosphatase (AP)-labeled goat anti-rat IgG (PharMingen) was added, and the plates were processed as described above.

Statistical analysis

Statistical analysis of the titration ELISA was conducted using the Mann-Whitney rank test, and the statistical analysis of the DNA fragmentation assay and the antitumor experiments was done using a two-tailed Student t test. For all cases, results were regarded significant if p values were ≤0.05.
fluorescence intensity was seen, which suggests that they have the same affinity for HER2/neu. No nonspecific binding to CT26 that does not express HER2/neu was observed (Fig. 3, E and F).

**Proliferation assay**

Anti-HER2/neu IgG3-(GM-CSF) was able to specifically stimulate the proliferation of the GM-CSF-dependent cell line FDC-P1. The proliferative response to equimolar GM-CSF concentrations of either rmGM-CSF or the anti-HER2/neu IgG3-(GM-CSF) fusion protein was similar (Fig. 4). No proliferation was detected when cells were incubated with the same amount of anti-HER2/neu IgG3 (data not shown). The GM-CSF activity of anti-HER2/neu IgG3-(GM-CSF) present in culture supernatants was similar to that of purified protein, indicating that the low pH used for elution from protein A does not reduce GM-CSF activity (data not shown).

**Macrophage-mediated cytotoxicity**

Two assays were used to examine the ability of anti-HER2/neu IgG3-(GM-CSF) to augment macrophage-mediated killing of tumor cells. Tumor cells and the macrophage cell line J774.2 were incubated for 24 h in the presence of 5 µg/ml anti-HER2/neu IgG3 or the molar equivalent of anti-HER2/neu IgG3-(GM-CSF). Equivalent tumor cell lysis was seen with both proteins, indicating that the Fc region of the fusion protein can be bound by the macrophage receptors to elicit ADCC (Fig. 5A). The tumor cell lysis observed with the incubation of anti-HER2/neu IgG3 or anti-HER2/neu IgG3-(GM-CSF) was statistically significant when compared with the results obtained with the incubation of the effector and target cells in absence of the Abs (p < 0.05). In the second assay, effector cells were incubated with 6.72 × 10^{-2} µg/ml anti-HER2/neu IgG3-(GM-CSF) or anti-HER2/neu IgG3, washed to remove unbound Ab or fusion protein, and then incubated with labeled target cells for 24 h. Anti-HER2/neu IgG3-(GM-CSF)-treated J774.2 cells were significantly (p < 0.0002) more effective in lysing tumor cells than the effector cells activated in presence of anti-HER2/neu IgG3 (Fig. 5B) which were similar to nonactivated effector cells added to labeled cells in the absence of Abs. Therefore, the GM-CSF in the fusion protein retains the ability to mediate macrophage activation.

**Half-life**

The half-life of 125I-labeled anti-HER2/neu IgG3 and anti-HER2/neu IgG3-(GM-CSF) was examined in BALB/c mice. Mice were injected i.v. via the lateral tail vein with 1 µCi (0.5 µg) 125I-labeled protein, and the residual radioactivity measured using a
mouse whole body counter. Anti-HER2/neu IgG3 exhibited a half-life of 110 h, similar to what had previously been observed with chimeric IgG3 (46) (Fig. 6). Anti-HER2/neu IgG3-(GM-CSF) cleared more rapidly with a half-life of 2 h, indicating that fusion of the murine GM-CSF to the human anti-HER2/neu IgG3 significantly decreases the half-life. However, because we plan to treat the mice with a much higher dose (20 mg) of anti-HER2/neu IgG3-(GM-CSF), we also studied the half-life when this amount of protein was injected by mixing 20 mg cold anti-HER2/neu IgG3-(GM-CSF) with 1 mCi (0.5 mg) 125I-labeled anti-HER2/neu IgG3-(GM-CSF) before injection. Increasing the quantity of injected anti-HER2/neu IgG3-(GM-CSF) injected increased the half-life 5- to 6-fold (10–12 h) (Fig. 6). Although results shown in Fig. 6 represent the mean of only two mice per group, similar results were obtained when this experiment was repeated (data not shown).

Sera obtained from each mouse 2, 4, and 12 h after injection were fractionated without reduction on SDS-PAGE and examined by autoradiography. The radioactivity was present at the position expected for intact protein, with the intensity of the band correlating with the residual radioactivity determined by whole body counting.

Biodistribution

Groups of four mice injected i.v. via the lateral tail vein with 1 mCi 125I-labeled anti-HER2/neu IgG3-(GM-CSF) were euthanized 4 h (time equivalent to two half-lives of the injected protein) or 16 h after injection. Increasing the quantity of injected anti-HER2/neu IgG3-(GM-CSF) injected increased the half-life 5- to 6-fold (10–12 h) (Fig. 6). Although results shown in Fig. 6 represent the mean of only two mice per group, similar results were obtained when this experiment was repeated (data not shown). Sera obtained from each mouse 2, 4, and 12 h after injection were fractionated without reduction on SDS-PAGE and examined by autoradiography. The radioactivity was present at the position expected for intact protein, with the intensity of the band correlating with the residual radioactivity determined by whole body counting.

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Biodistribution

Groups of mice injected i.v. via the lateral tail vein with 1 μCi 125I-labeled anti-HER2/neu IgG3-(GM-CSF) were euthanized 4 h (time equivalent to two half-lives of the injected protein) or 16 h after injection. Increasing the quantity of injected anti-HER2/neu IgG3-(GM-CSF) injected increased the half-life 5- to 6-fold (10–12 h) (Fig. 6). Although results shown in Fig. 6 represent the mean of only two mice per group, similar results were obtained when this experiment was repeated (data not shown).

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after injection. Various organs and blood were collected and weighed, and radioactivity was measured using a gamma counter. Four hours after its injection anti-HER2/neu IgG3-(GM-CSF) shows targeting to the spleen, followed by the kidneys, liver, and lungs (Fig. 7A). By 16 h after the injection, most of anti-HER2/neu IgG3-(GM-CSF) had cleared with some radioactivity remaining in the spleen, kidneys, and blood. Splenic uptake may reflect the large number of GM-CSF receptor-bearing cells in this organ. The presence of radioactivity in the kidneys and liver, sites of degradation and elimination, is consistent with the rapid elimination of anti-HER2/neu IgG3-(GM-CSF).

Tumor targeting

To examine the tumor targeting capability of anti-HER2/neu IgG3-(GM-CSF), BALB/c mice were injected with 10⁶ CT26 and CT26-HER2/neu tumor cells in the left and right flanks, respectively. Seven days after tumor injection when tumors were ~1.0 cm in diameter, groups of three mice were injected i.v. via the lateral tail vein with 6 μCi ¹²⁵I-labeled anti-HER2/neu IgG3-(GM-CSF). The mice were euthanized 12 h later, the tumors and blood were removed and weighed, and the ¹²⁵I-labeled protein present was measured by a gamma counter. In all mice, enhanced localization of ¹²⁵I-labeled anti-HER2/neu IgG3-(GM-CSF) was seen in the CT26-HER2/neu tumor compared with CT26 that did not express HER2/neu (Fig. 8). These data indicate that anti-HER2/neu IgG3-(GM-CSF) is able to specifically target HER2/neu-expressing cells.

Antitumor activity

To investigate in vivo antitumor activity, 10⁶ CT26-HER2/neu cells were injected s.c. into the right flank of BALB/c mice. Beginning the next day, mice were randomized, and groups of eight received five daily i.v. injections of 0.25 ml PBS containing 20 μg anti-HER2/neu IgG3-(GM-CSF), the equivalent molar amount of anti-HER2/neu IgG3, or nothing. Injection of anti-HER2/neu IgG3-(GM-CSF) results in a significant retardation in the tumor growth in most of the mice as compared with the respective controls of PBS or anti-HER2/neu IgG3 (Fig. 9, Experiment 1). When the experiment was repeated similar results were obtained (Fig. 9, Experiment 2). When the data of Experiments 1 and 2 were pooled, treatment with anti-HER2/neu IgG3-(GM-CSF) was found to result in highly significant antitumor activity (p < 0.02) for all the observed points (Table I). There was no statistically significant difference in tumor volume between the groups injected with PBS and anti-HER2/neu IgG3.

Murine Ab response to HER2/neu and human IgG3

Sera from all mice in Experiment 2 were analyzed for the presence of Abs recognizing the TAA HER2/neu and the human IgG3 Ab used for treatment. Mice treated with anti-HER2/neu IgG3-(GM-CSF) exhibited a significantly increased Ab response to both HER2/neu (p < 0.04) and human IgG3 (p < 0.001) compared with mice treated with either PBS or anti-HER2/neu IgG3 (Table II).
Isotype of murine Ab response

To further characterize the Ab response, the relative levels of the different isotypes present in the serum of anti-HER2/neu IgG3-(GM-CSF) and anti-HER2/neu IgG3-treated mice were determined (Fig. 10). Mice treated with anti-HER2/neu IgG3-(GM-CSF) showed significantly higher levels of all isotypes (with the exception of IgG3) recognizing human IgG3 when compared with anti-HER2/neu IgG3 treated mice (Fig. 10A). The increase in Abs of the γ2a and γ1 isotypes suggests activation of both Th1- and Th2-mediated responses against this Ag, respectively. When Abs directed against HER2/neu were examined (Fig. 10B), animals treated with anti-HER2/neu IgG3-(GM-CSF) showed an increase in γ2b and γ1 but not γ3 and γ2a compared with animals treated with anti-HER2/neu IgG3. Thus, the increased Ab response to HER2/neu was predominantly of the isotypes characteristic of the Th2 response.

Discussion

In an attempt to improve the clinical efficacy of anti-HER2/neu based therapies, we have developed an alternative approach in which a human IgG3 containing the variable regions of trastuzumab (Herceptin, Genentech, San Francisco, CA) has been genetically fused to potent immunostimulatory molecules such as the cytokine IL-12 (47) and the costimulatory molecule B7.1 (41). In an attempt to improve the clinical efficacy of anti-HER2/neu antibodies, we have developed an alternative approach in which a human IgG3 containing the variable regions of trastuzumab (Herceptin, Genentech, San Francisco, CA) has been genetically fused to potent immunostimulatory molecules such as the cytokine IL-12 (47) and the costimulatory molecule B7.1 (41). This fusion protein, human IgG3-(GM-CSF), was designed to include a fusion with the important cytokine GM-CSF.

A number of factors were considered in the design of our anti-HER2/neu IgG3-(GM-CSF) fusion protein. Human IgG3 was chosen because its extended hinge region should provide spacing and flexibility, thereby facilitating simultaneous Ag and receptor binding (48, 49). IgG3 is also effective in complement activation (50) and binds FcγRs (51). GM-CSF was used because of its potent immunostimulating properties and ability to serve as a strong potentiator of tumor vaccines (26–30). Although our long-term goal is the production of Ab fusion proteins for therapeutic use in humans, human GM-CSF is not active in mice (35). Therefore we used murine GM-CSF in our fusion protein so that we could perform in vivo studies using immune competent mice. We found that anti-HER2/neu IgG3-(GM-CSF) retains the ability to bind HER2/neu while the murine GM-CSF attached to the carboxyl terminus of each heavy chain remains active.

In addition to the Ab-induced down-regulation of HER2/neu expression ADCC has been proposed as a possible mechanism for the clinical response observed with trastuzumab (15). Indeed, recent studies have indicated that ADCC is an important effector mechanism for Ab-mediated tumor rejection (52). Fusion of GM-CSF to the carboxyl terminus of Cγ3 did not interfere with the ability of Ab to mediate ADCC (Fig. 5A). In addition, preincubation of macrophages with a very low concentration of anti-HER2/neu IgG3-(GM-CSF) results in a significant activation of macrophage-mediated cytotoxicity as compared with anti-HER2/neu IgG3 (Fig. 5B). In this latter experiment Abs were not added to the E:T mixture, suggesting that preincubation of macrophage with anti-HER2/neu IgG3-(GM-CSF) results in the activation of ADCC. However, because the effector cells were preincubated with anti-HER2/neu IgG3-(GM-CSF), the possibility of ADCC mediated by Ab-coated effector cells cannot be excluded.

A recombinant fusion protein with a human-mouse chimeric IgG1 specific for B cell malignancies fused to human GM-CSF (chCLL-1/1-GM-CSF) showed enhanced ADCC activity using human mononuclear cells compared with Ab (chCLL-1) alone (53). It is therefore possible that an anti-HER2/neu IgG3-(GM-CSF) containing human GM-CSF will exhibit superior antitumor activity. In addition directing GM-CSF to the tumor microenvironment

FIGURE 8. Tumor targeting of anti-HER2/neu IgG3-(GM-CSF). CT26-HER2/neu and CT26 cells (10⁶) were injected separately into the right and left flanks of three BALB/c mice. After 1 wk, when the tumor diameter was ~1.0 cm, groups of three mice were injected i.v. via the lateral tail vein with 125I-labeled anti-HER2/neu IgG3-(GM-CSF). Mice were euthanized 12 h after injection. Blood and tumors were collected and weighed, and radioactivity was measured by a gamma counter. Data are presented as %ID/g tumor.

FIGURE 9. Antitumor activity of anti-HER2/neu IgG3-(GM-CSF) and anti-HER2/neu IgG3. 10⁶ CT26-HER2/neu cells were injected s.c. into the right flank of BALB/c mice. Beginning the next day, groups of eight mice received five daily i.v. injections of 0.25 ml PBS containing 20 μg anti-HER2/neu IgG3-(GM-CSF), the equivalent molar amount of anti-HER2/neu IgG3, or nothing. Tumor growth was measured with a caliper every 3 days until day 15. The volume was calculated for each mouse of each treatment group, PBS (A), anti-HER2/neu IgG3 (B), and anti-HER2/neu IgG3-(GM-CSF) (C). Experiments 1 and 2 were conducted under identical conditions but at different time.
jurious cytokine exposure to normal tissues should be minimized.

It has yet to be determined in patients whether the higher doses saturated the available GM-CSF receptors. It has yet to be determined in patients whether the rapid clearance is through the GM-CSF receptor than does human GM-CSF (59), which may lead to more rapid clearance. A GM-CSF fusion protein specific for the murine transferrin receptor had a half-life of ~1.8 h (60). In this case, it is likely that the Ab fusion proteins were rapidly cleared by the ubiquitous transferrin receptor (61).

We have found that treatment with anti-HER2/neu IgG3-(GM-CSF) causes a significant retardation in the growth of s.c. CT26-HER2/neu tumors under conditions in which anti-HER2/neu IgG3 failed to confer protection. Our data are consistent with earlier experiments in which ch17217-(murine GM-CSF) specific for the murine transferrin receptor suppressed the development of pulmonary metastasis in five of eight immunocompetent mice injected with CT26. However, the control of Ab alone (ch17217) was not included in these earlier studies, making it impossible to distinguish the role of the Ab from that of GM-CSF (60). In those studies as well as our own, the control of Ab plus GM-CSF is also absent. Unfortunately, we did not have enough free GM-CSF available to include it as a control. Nevertheless, ours is the first report showing that an antitumor Ab-(GM-CSF) fusion protein shows a significant antitumor activity under conditions in which the Ab alone (anti-HER2/neu IgG3) fails to confer protection.

Several factors could explain our failure to obtain complete tumor remission. The dose, route, and schedule of treatment (daily i.v. injection of 20 μg for 5 days) may not be the optimal and/or

### Table I. Mean tumor volumes and statistical significance

<table>
<thead>
<tr>
<th>Days After Challenge</th>
<th>Mean Tumor Volumes*</th>
<th>Significanceb</th>
</tr>
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<tr>
<td></td>
<td>PBS</td>
<td>IgG3</td>
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<td>6</td>
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</tr>
<tr>
<td>12</td>
<td>578.2</td>
<td>631.8</td>
</tr>
<tr>
<td>15</td>
<td>1041.8</td>
<td>1155.6</td>
</tr>
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</table>

* CT26-HER2/neu cells (10⁶) were injected s.c. into the right flank of BALB/c mice. Beginning the next day, groups of eight mice received five daily i.v. injections of 0.25 ml PBS containing 20 μg anti-HER2/neu IgG3-(GM-CSF), the equivalent molar amount of anti-HER2/neu IgG3, or nothing. Tumor growth was measured with a caliper every 3 days until day 15, and the volume was calculated for each mouse of each treatment group. The experiment was conducted twice under identical conditions. Mean tumor volumes represent the average tumor volume for each treatment group when the data of the two experiments were pooled.

## Table II. Murine anti-human HER2/neu and anti-human IgG3 titers

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Anti-HER2/neu Titters</th>
<th>Anti-Human IgG Titters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS</td>
<td>IgG3</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>8</td>
<td>450</td>
<td>450</td>
</tr>
</tbody>
</table>

* Groups of eight mice injected s.c. with 10⁶ CT26-HER2/neu cells were treated beginning the next day with five daily i.v. injections of 0.25 ml PBS containing 20 μg anti-HER2/neu IgG3-(GM-CSF), the equivalent molar amount of anti-HER2/neu IgG3, or nothing. Mice were bled 15 days after the injection of the tumor cells, and the sera were analyzed by a titration ELISA using plates coated with the ECD HER2 or human IgG3. The presence of Abs was detected using AP-labeled anti-mouse IgG. Values represent the average of duplicate dilutions of serum required to yield an optical density of 0.1 (410 nm) after 1 h of incubation.

** N/A, Not applicable.
the tumor model may not be ideal for this particular study. In addition, we found that treatment with anti-HER2/neu IgG3-(GM-CSF) increases the endogenous humoral immune response against the human HER2/neu (39). Because we have evidence that endogenous Abs may inhibit the binding of recombinant anti-HER2/neu IgG3 to the tumor cells (39), this enhanced Ab response in anti-HER2/neu IgG3-(GM-CSF)-treated mice may further interfere with the binding of the anti-HER2/neu IgG3-(GM-CSF) to the cancer cells resulting in less effective antitumor activity. However, this may be a limitation only in the studies using murine tumors in which the expression of HER2/neu is not related to cell survival. In patients, the ability of anti-HER2/neu IgG3-(GM-CSF) to elicit a strong humoral immune response may be advantageous because Abs targeting HER2/neu on human tumors appear to directly inhibit their growth (15). Therefore, increasing the immune response using cytokines such as GM-CSF may facilitate tumor eradication. In fact, immunization using GM-CSF fused to the Ig expressed by a lymphoma can cause regression of the lymphoma in mice (62). The dramatically increased Ab response to the TAA HER2/neu is consistent with effective tumor targeting by anti-HER2/neu IgG3-(GM-CSF).

The isotype of the humoral immune response against human IgG and human HER2/neu suggests that anti-HER2/neu IgG3-(GM-CSF) has the ability to enhance both Th1 (T cell-directed) and Th2 (B cell-directed) immune responses. However, we do not know the effector mechanism responsible for the antitumor activity of anti-HER2/neu IgG3-(GM-CSF) observed in animals bearing CT26-HER2/neu tumors. Although ADCC mediated by effector cells such as macrophages, eosinophils, and NK cells is a possibility, CD8+ (27) and CD4+ (27, 30) cells may also play a role in that they have been shown to be necessary for protection against tumor cell challenge in mice vaccinated with irradiated GM-CSF-secreting tumor cells.

In conclusion, our results suggest that an anti-HER2/neu IgG3-(GM-CSF) fusion protein containing human GM-CSF may be effective in patients with tumors overexpressing HER2/neu. The combination of an anti-HER2/neu Ab with GM-CSF yields a protein with the potential to eradicate tumor cells by a number of mechanisms including the down-regulation of HER2/neu expression, ADCC, and the stimulation of a strong antitumor immune response through the immunostimulatory activity of GM-CSF. In addition, the anti-HER2/neu IgG3-(GM-CSF) fusion protein may be effective against tumor cells that express a truncated form of HER2/neu, lacking the receptor function rendering them particularly resistant to anti-HER2/neu Ab therapy (14). Because of the ability of GM-CSF to elicit an immune response to associated Ags, it is also possible that association of anti-HER2/neu IgG3-(GM-CSF) with soluble ECDHER2/neu shed by tumor cells will enhance the antitumor immune response.

Finally, we would like to stress that anti-HER2/neu IgG3-(GM-CSF) would not be a replacement for Herceptin but instead would provide an alternative therapy to be used in combination with the Ab or other anticaner approaches. These approaches might include chemotheraphy or other anti-HER2/neu Ab fusion proteins such as anti-HER2/neu with the costimulator B7.1 (41) or the cytokine IL-12 (47). The availability of more than one Ab fusion protein will allow us to explore potential synergistic effects that may be obtained from manipulating the immune response.

**Acknowledgments**

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**References**


