The Journal of Immunology

RESEARCH ARTICLE | NOVEMBER 01 2000

Recombinant Anti-Human HER2/*neu* IgG3-(GM-CSF) Fusion Protein Retains Antigen Specificity and Cytokine Function and Demonstrates Antitumor Activity [1] FREE

Jay S. Dela Cruz; ... et. al

J Immunol (2000) 165 (9): 5112–5121.
https://doi.org/10.4049/jimmunol.165.9.5112

Recombinant Anti-Human HER2/*neu* IgG3-(GM-CSF) Fusion Protein Retains Antigen Specificity and Cytokine Function and Demonstrates Antitumor Activity[1]

Jay S. Dela Cruz, K. Ryan Trinh, Sherie L. Morrison, and Manuel L. Penichet[2]

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 $p185^{HER2}$ 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 (8), endometrial (9), and salivary gland cancers (10). The increased occurrence of visceral metastasis and micrometastatic 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 rhumAb HER2, directed at the extracellular domain of HER2/*neu* (ECD^{HER2}) (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

Departments of Microbiology and Molecular Genetics, and The Molecular Biology Institute, University of California, Los Angeles, CA 90095

Received for publication March 28, 2000. Accepted for publication August 9, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by Grant 3CB-0245 from the University of California Breast Cancer Research Program, Susan G. Komen Breast Cancer Foundation Grant 9855, Department of Defense Breast Cancer Research Program Grant BC980134, Tumor Immunology Training Grant 5-T32-CA09120-24 from the National Cancer Institute (National Institutes of Health), and Cancer Center Core Grant CA-16042 (from the University of California at Los Angeles).

[2] Address correspondence and reprint requests to Dr. Manuel L. Penichet, Department of Microbiology and Molecular Genetics, University of California, 405 Hilgard Avenue, Los Angeles, CA 90095-1489. E-mail address: penichet@microbio.ucla.edu

[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; ECD^{HER2}, 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.

0022-1767/00/$02.00

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 fatality (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 TAAs 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 intrarectal 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 cDNA-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% CO_2.

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-C_H3-(GM-CSF)*

The DNA encoding the variable light (V_L) and heavy (V_H) chain domains of the humanized Ab hum4D5-8 (13) (15) or rhuMAb HER2 (14, 16) (generously provided by Paul Carter, Genentech) had previously been cloned into mammalian expression vectors for human κ 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′-CCCCTCGCGAGCGCACCCAC CCGCTCACCC-3′ and the antisense primer 5′-CCGAATTCGTTAAC CTTTTTGGACTGGTTTTTTGCATTC-3′.

The PCR product was digested with *Nru*I/*Eco*RI and cloned in the vector pAT3462 (previously developed in our laboratory) digested with *Ssp*I/*Eco*RI, yielding the vector pAT1791 (Fig. 1). The plasmids pAT6611, pAH4874 (both previously developed in our laboratory), and pAT1791 were digested with *Eco*RV/NsiI, *Eco*RV/*Bam*HI, and *Nsi*I/*Bam*HI, respectively. The fragments containing the DNA encoding for anti-HER2/*neu* V_H 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-C_H3-(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 mycophenolic acid-stable transfectants. These were screened for L-chain secretion by ELISA (42). The heavy chain expression vector pAH1792 was used 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 [^{35}S]methionine (ICN, Irvine, CA), and the fusion protein was immunoprecipitated using rabbit anti-human IgG and a 10% suspension of staphylococcal protein A (IgGSorb, The Enzyme Center, Malden, MA) 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 bicinchoninic 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* (10^6) 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. Recombinant anti-HER2/*neu* IgG3 (41) and recombinant anti-DNS IgG3 Abs were used as positive and negative isotype-matched controls, respectively. Cells were washed and incubated for 2 h at 4°C with 0.5 μg biotinylated goat anti-human IgG (PharMingen, 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 (PharMingen) in a volume of 0.1 ml PBS plus 2% of 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.

Proliferation 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 $ED_{50} \leq 0.2$ ng/ml (Chemicon, Temecula, CA) reconstituted using deionized water following the manufacturer's recommendations and stored at −20°C was used as reference standard. Serial 1:2 dilutions of equivalent molar concentrations of rmGM-CSF and anti-HER2/*neu* IgG3-(GM-CSF) were made in RPMI 1640 + 10% FBS, over a range of 2 ng/ml to 16 pg/ml. Similarly, serial 1:2 dilutions of control anti-HER2/*neu* IgG3 were also included with a concentration equivalent to the Ab portion of anti-HER2/*neu* IgG3-(GM-CSF). 50 μl (5000 cells/well) FDC-P1 myeloid cells in RPMI 1640 + 10% FBS were mixed with 50 μl serial dilutions of rmGM-CSF, anti-HER2/*neu* IgG3-(GM-CSF), anti-HER2/*neu* IgG3, or medium in quadruplicate in a flat-bottom 96-well tissue culture plate (Costar, Corning, NY). After 48 h of culture at 37°C, 5% CO_2, proliferation was measured using the Cell Titer 96 aqueous nonradioactive colorimetric assay (Promega, Madison, WI), and plates were read at 490 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 [^{3}H]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 J774.2 macrophage effector cell 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 6.72×10^{-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^{-2} 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 1×10^4 [^{3}H]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 [^{3}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 by the formula: [(cpm control − cpm test)/cpm control)] × 100; where cpm control represents ^{3}H measured in the wells containing target cells and anti-HER2/*neu* IgG3, anti-HER2/*neu* IgG3-(GM-CSF), or medium but lacking J774.2 macrophage cells. cpm test represents wells containing target cells in the presence of either effector cells preincubated with anti-HER2/*neu* IgG3 or anti-HER2/*neu* IgG3-(GM-CSF) or neither and Abs (anti-HER2/*neu* IgG3 or anti-HER2/*neu* IgG3-(GM-CSF)). All assays were done in quadruplicate.

Half-life

Anti-HER2/*neu* IgG3-(GM-CSF) was iodinated to ~2 μCi/μg with ^{125}I using Iodo-Beads (Pierce) according to manufacturer's protocol. Mice were injected i.v. via the lateral tail vein with 1 μCi ^{125}I-labeled proteins alone or mixed with 20 μg cold anti-HER2/*neu* IgG3-(GM-CSF). At various intervals after injection of ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF), residual radioactivity was measured using a mouse whole body counter (Wm. 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.

Biodistribution

Groups of 4 mice were sacrificed 4 or 16 h after the i.v. injection of 1 μCi (0.5 μg) ^{125}I-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 percent 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 (10^6 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 ^{125}I-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 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 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 ECD^{HER2} (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, IgG1, 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.

Results

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

The strategy for the construction and expression of anti-HER2/*neu* IgG3-C$_H$3-(GM-CSF) is illustrated in Fig. 1. Clones expressing anti-HER2/*neu* IgG3-C$_H$3-(GM-CSF) were identified by ELISA and biosynthetically labeled by growth in the presence of [^{35}S]methionine. Labeled secreted protein was immunoprecipitated using rabbit anti-human IgG and analyzed by SDS-PAGE under reducing and nonreducing conditions. The anti-HER2/*neu* IgG3-C$_H$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*A*). 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. 2*B*) 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_2L_2 form 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

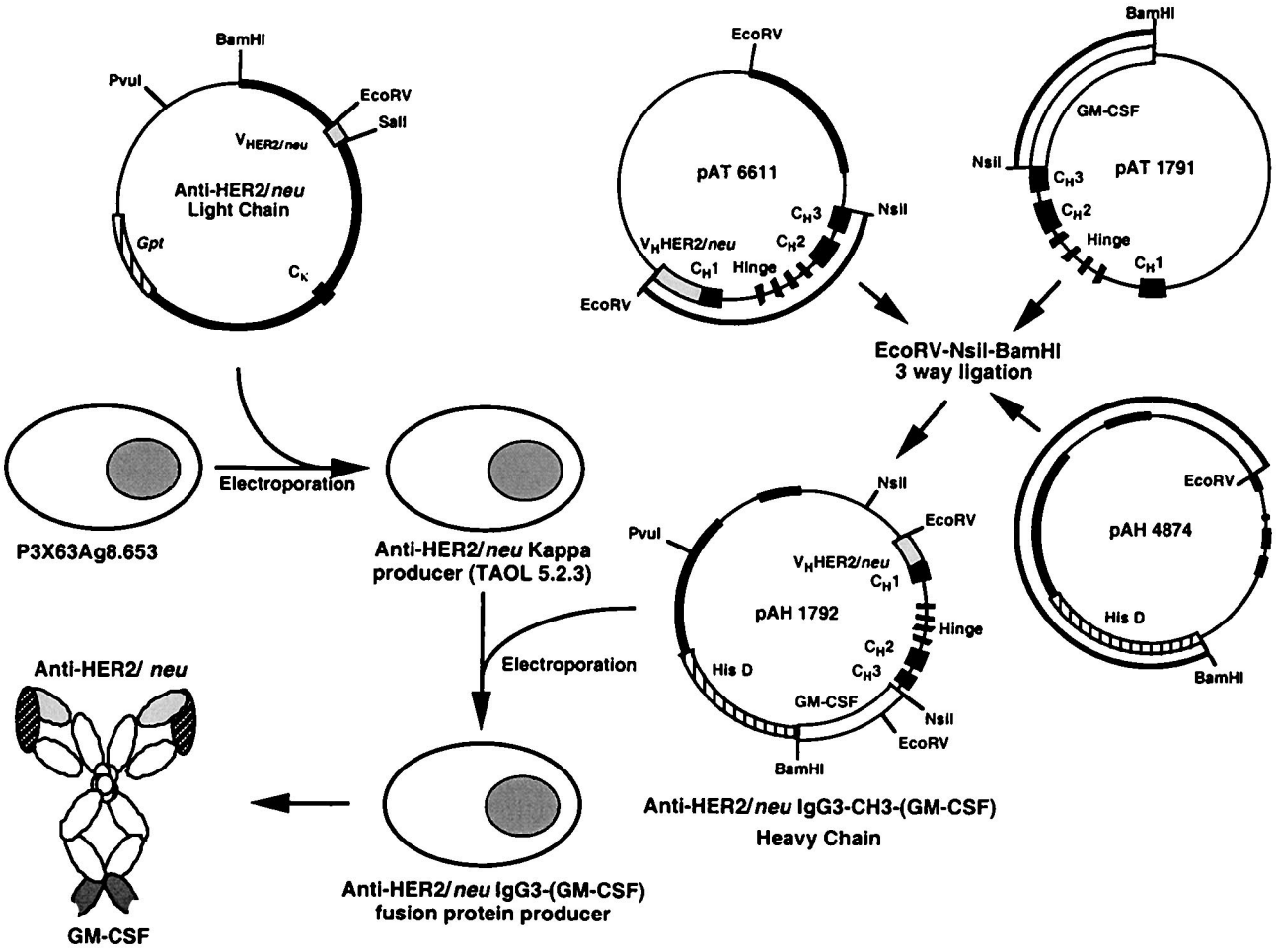

FIGURE 1. Construction and expression of anti-HER2/*neu* IgG3-(GM-CSF). The expression vector for anti-HER2/*neu* IgG3-(GM-CSF), pAH1792, was constructed by three-way ligation of the fragments containing the V_H anti-HER2/*neu* and constant IgG3 regions from pAT6611, the expression vector backbone from pAH4874, and GM-CSF from pAT1791. A solid line outside the plasmid indicates the fragment used in the three-way ligation. TAOL 5.2.3, a transfectant of P3X63Ag8.653 expressing a light chain with the anti-HER2/*neu* variable region, was used as a recipient for transfection of the anti-HER2/*neu* IgG3-(GM-CSF) heavy chain expression vector pAH1792.

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. 5*A*). 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. 5*B*) 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 ^{125}I-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) ^{125}I-labeled protein, and the residual radioactivity measured using a

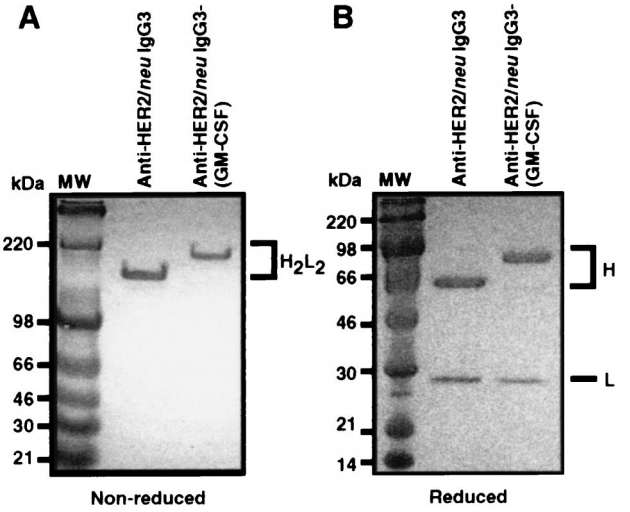

FIGURE 2. SDS-PAGE analysis of anti-HER2/*neu* IgG3-(GM-CSF). Secreted anti-HER2/*neu* IgG3-(GM-CSF) was purified from culture supernatants using protein A immobilized on Sepharose 4B fast flow and analyzed by SDS-PAGE under nonreducing (*A*) and reducing (*B*) conditions. Included for comparison is anti-HER2/*neu* IgG3 without attached GM-CSF. The positions of the m.w. standards are indicated at the left sides.

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 μg) of anti-HER2/*neu* IgG3-(GM-CSF), we also studied the half-life when this amount of protein was injected by mixing 20 μg cold anti-HER2/*neu* IgG3-(GM-CSF) with 1 μCi (0.5 μg) ^{125}I-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).

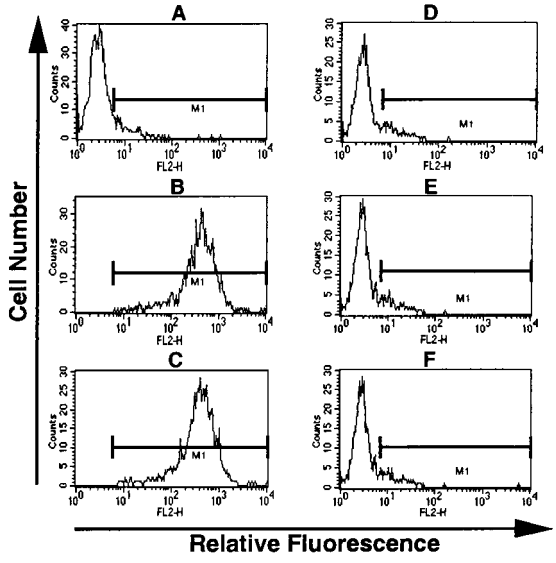

FIGURE 3. Flow cytometry demonstrating the specificity of anti-HER2/*neu* IgG3-(GM-CSF) for the HER2/*neu* expressed on the surface of CT26-HER2/*neu*. CT26-HER2/*neu* (*A–C*) or the non-HER2/*neu*-expressing parental cell line CT26 (*D–F*) were stained with anti-DNS human IgG3 (*A* and *D*), anti-HER2/*neu* human IgG3 (*B* and *E*) or anti-HER2/*neu* IgG3-(GM-CSF) (*C* and *F*), followed by biotinylated goat anti-human IgG and PE-labeled streptavidin. FL2-H, Fluorescence.

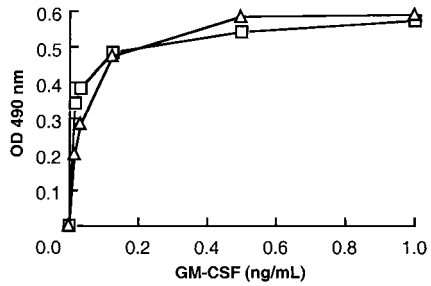

FIGURE 4. Bioactivity assay. FDC-P1 cells were incubated with various concentrations of rmGM-CSF (□) or anti-HER2/*neu* IgG3-(GM-CSF) (△). The concentration of anti-HER2/*neu* IgG3-(GM-CSF) was adjusted to the GM-CSF portion of the fusion protein obtaining equivalent molar concentrations of rmGM-CSF and anti-HER2/*neu* IgG3-(GM-CSF). Proliferation was measured by a colorimetric assay and read at 490 nm. All results are expressed as mean OD_{490} of quadruplicate wells with a SD of <20% for each concentration.

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 μCi ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF) were euthanized 4 h (time equivalent to two half-lives of the injected protein) or 16 h

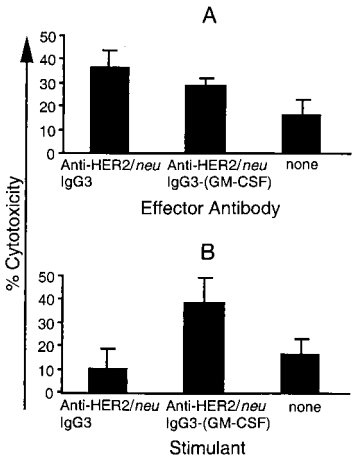

FIGURE 5. Macrophage-mediated cytotoxicity. *A*, A total of 1×10^4 ^{3}H-labeled CT26-HER2/*neu* target cells were cultured for 24 h with anti-HER2/*neu* IgG3 (5 μg/ml), the equivalent molar concentration of anti-HER2/*neu* IgG3-(GM-CSF), or nothing in the presence of J774.2 macrophage effector cells at an E:T ratio of 10. *B*, Effector cells were preincubated for 24 h with anti-HER2/*neu* IgG3-(GM-CSF) (6.72×10^{-2} μg/ml), the equivalent molar concentration of anti-HER2/*neu* IgG3, or nothing; washed; and then incubated with 1×10^4 ^{3}H-labeled CT26-HER2/*neu* target cells for 24 h. For both assays, intact DNA from live target cells was collected by a cell harvester, and radioactivity was measured using a scintillation counter. Bars represent the SD of quadruplicate samples.

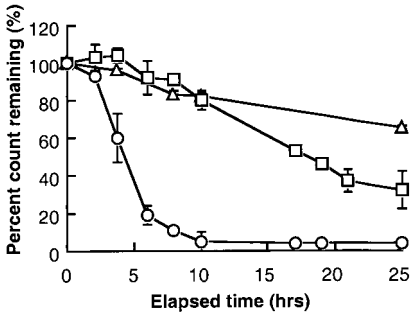

FIGURE 6. Half-life of anti-HER2/*neu* IgG3-(GM-CSF) and anti-HER2/*neu* IgG3. Groups of two mice were injected i.v. via the lateral tail vein with 1 μCi (0.5 μg) ^{125}I-labeled anti-HER2/*neu* IgG3 (△), anti-HER2/*neu* IgG3-(GM-CSF) (○), or 1 μCi (0.5 μg) ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF) mixed with 20 μg cold anti-HER2/*neu* IgG3-(GM-CSF) (□). At various intervals after injection of the ^{125}I-labeled protein, residual radioactivity was measured using a mouse whole body counter. The results represent the mean of two mice. Bars represent the range of values obtained.

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. 7*A*). 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 10^6 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 ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF). The mice were euthanized 12 h later, the tumors and blood were removed and weighed, and the ^{125}I-labeled protein present was measured by a gamma counter. In all mice, enhanced localization of ^{125}I-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, 10^6 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 ($p \leq 0.02$) 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).

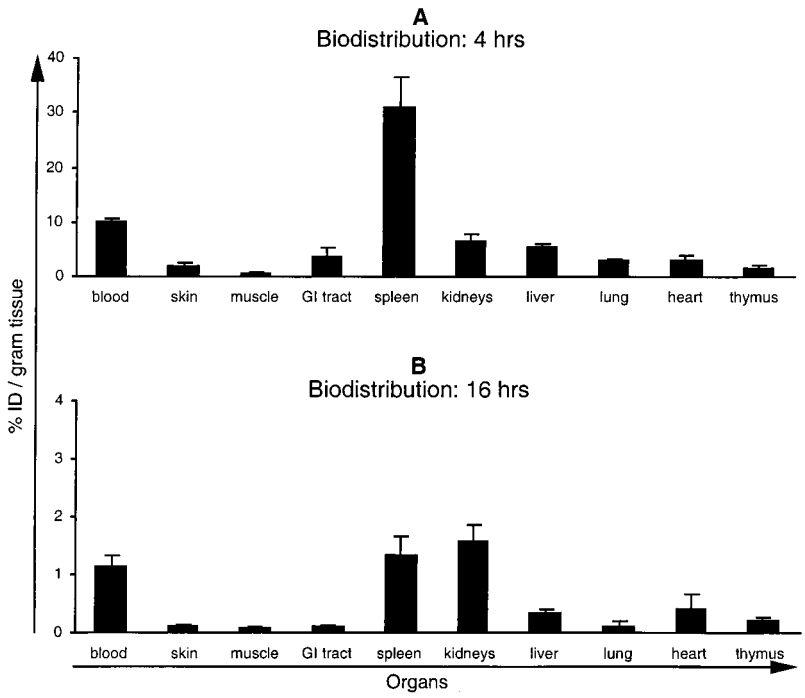

FIGURE 7. Biodistribution of anti-HER2/*neu* IgG3-(GM-CSF). Two groups of four mice were injected i.v. via the lateral tail vein with 1 μCi (0.5 μg) ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF), and mice were euthanized after 4 h, which is the equivalent of two half-lives for the injected dose or after 16 h. Various organs and blood were collected and weighed, and radioactivity was measured using a gamma counter. Data are presented as %ID/g tissue. GI, Gastrointestinal. Bars represent the SD of the data obtained.

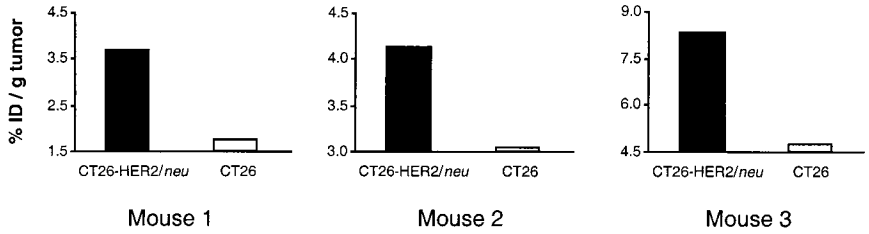

FIGURE 8. Tumor targeting of anti-HER2/*neu* IgG3-(GM-CSF). CT26-HER2/*neu* and CT26 cells (10^6) were injected separately into the right and left flanks of three BALB/c mice. After 1 wk, when the tumor diameter was ~1.0 cm, groups of three mice were injected i.v. via the lateral tail vein with ^{125}I-labeled 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.

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. 10*A*). 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. 10*B*), 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 the present study, we expand this family of anti-HER2/*neu* Ab fusion proteins 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 C_H3 did not interfere with the ability of Ab to mediate ADCC (Fig. 5*A*). 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. 5*B*). 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/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

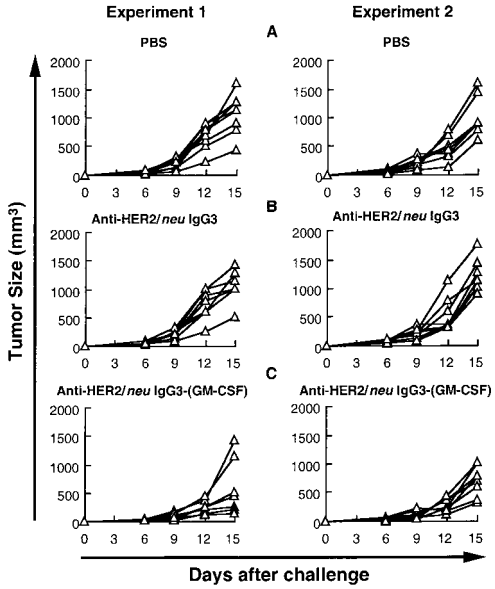

FIGURE 9. Antitumor activity of anti-HER2/*neu* IgG3-(GM-CSF) and anti-HER2/*neu* IgG3. 10^6 CT26-HER2/*neu* cells were injected s.c. into the right flank of BALB/c mice. Beginning the next day, groups of eight mice received five daily i.v. injections of 0.25 ml PBS containing 20 μg anti-HER2/*neu* IgG3-(GM-CSF), 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.

Table I. *Mean tumor volumes and statistical significance*

Days After Challenge	Mean Tumor Volumes[a]			Significance[b]	
	PBS	IgG3	IgG3-(GM-CSF)	(*p*) 1	(*p*) 2
6	60.8	71	37.6	0.02	0.0006
9	211	224.5	110.5	0.0008	0.0003
12	578.2	631.8	264.9	0.0001	0.0001
15	1041.8	1155.6	655.3	0.0053	0.0002

[a] CT26-HER2/*neu* cells (10^6) 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 represents the average tumor volume for each treatment group when the data of the two experiments were pooled.

[b] Statistical analysis of 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. (*p*) 1 and (*p*) 2 represent the *p* obtained when mean tumor volumes of the group injected with anti-HER2/*neu* IgG3-(GM-CSF) were compared with PBS and anti-HER2/*neu* IgG3 controls, respectively.

using anti-HER2/*neu* IgG3-(GM-CSF) may lead to enhanced macrophage activation at the site of the tumor; in murine models, activated macrophages given locally and i.v. inhibit tumor growth and decrease metastatic development (54).

Systemic clearance of anti-HER2/*neu* IgG3-(GM-CSF) is rapid compared with anti-HER2/*neu* IgG3. This is consistent with observations with other Ab cytokine fusion proteins (55), demonstrating a dominant role for the attached cytokine in determining the pharmacokinetics of the fusion proteins. We believe that the rapid clearance of the Ab fusion protein is through the GM-CSF receptors on normal cells (35) such as splenic T cells, B cells, and macrophages (56). In fact, our biodistribution studies showed that anti-HER2/*neu* IgG3-(GM-CSF) is mainly localized in the spleen consistent with earlier reports for the site targeted by murine GM-CSF (57). Interestingly, we found a dose-dependent rate of clearance with rapid clearance ($t_{1/2}$ = 2 h) seen when 0.5 μg was injected and slower clearance ($t_{1/2}$ = 10–12 h) when 20 μg was injected. Possibly, the higher doses saturated the available GM-CSF receptors. It has yet to be determined in patients whether the kinetics of clearance of anti-HER2/*neu* IgG3-(GM-CSF) will depend on the dose administered, although in a clinical study using nonglycosylated human GM-CSF injected i.v., no clear relationship between dose and half-life was observed (58). Despite its rapid clearance, anti-HER2/*neu* IgG3-(GM-CSF) retains the capacity to effectively target the tumor. In fact, the rapid clearance may be beneficial in clinical applications in which potentially injurious cytokine exposure to normal tissues should be minimized.

A half-life of ~30 h has been reported for the chCLL-1/GM-CSF fusion protein injected i.p. (53). The difference in clearance rates between anti-HER2/*neu* IgG3-(GM-CSF) and chCLL-1/GM-CSF may be explained by the use of different doses, by the route of injection (i.v. and i.p. respectively) and/or by the nature of the GM-CSF which were murine and human, respectively. Murine GM-CSF has considerably higher affinity for the murine 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 II. *Murine anti-human HER2/*neu *and anti-human IgG3 titers*[a]

Mouse	Anti-HER2/*neu* Titers			Anti-Human IgG Titers		
	PBS	IgG3	IgG3-(GM-CSF)	PBS	IgG3	IgG3-(GM-CSF)
1	150	150	12,150	N/A[b]	450	4,050
2	12,150	450	4,050	N/A	450	36,450
3	450	450	1,350	N/A	150	4,050
4	450	450	1,350	N/A	150	4,050
5	1,350	450	4,050	N/A	450	12,150
6	450	450	450	N/A	150	4,050
7	50	150	4,050	N/A	150	1,350
8	450	450	1,350	N/A	150	4,050

[a] Groups of eight mice injected s.c. with 10^6 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.

[b] N/A, Not applicable.

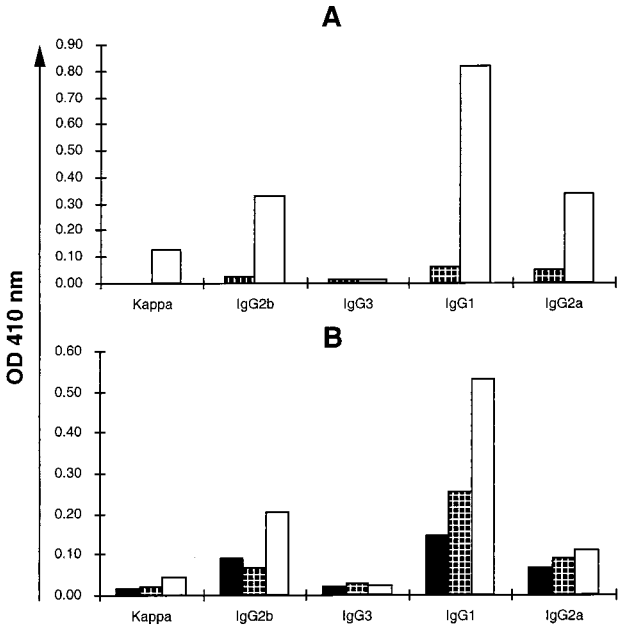

FIGURE 10. Isotype profile of Abs specific for HER2/*neu* and human IgG3. Pooled sera from mice treated with PBS (■), anti-HER2/*neu* IgG3 (▩), or anti-HER2/*neu* IgG3-(GM-CSF) (□) were analyzed by ELISA for Abs of different isotypes recognizing either anti-HER2/*neu* IgG3 (*A*) or ECD^{HER2} (*B*).

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 ECD^{HER2} 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 ECD^{HER2} 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 anticancer approaches. These approaches might include chemotherapy 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

We thank Dr. Mi-Hua Tao for the cDNA of murine GM-CSF, Dr. Paul Carter for providing the sequences of humanized humAb4D5 Ab, Dr. Matthew Scharff for the cell line J774.2, Dr. James D. Marks for ECD^{HER2}, and Dr. Donald Morrison for assistance with statistical analysis.

References

1. Coussens, L., T. L. Yang-Feng, Y. C. Liao, E. Chen, A. Gray, J. McGrath, P. H. Seeburg, T. A. Libermann, J. Schlessinger, U. Francke, et al. 1985. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. *Science 230:1132.*
2. Akiyama, T., C. Sudo, H. Ogawara, K. Toyoshima, and T. Yamamoto. 1986. The product of the human c-*erbB-2* gene: a 185-kilodalton glycoprotein with tyrosine kinase activity. *Science 232:1644.*
3. Stern, D. F., P. A. Heffernan, and R. A. Weinberg. 1986. p185, a product of the *neu* proto-oncogene, is a receptorlike protein associated with tyrosine kinase activity. *Mol. Cell. Biol. 6:1729.*
4. Slamon, D. J., G. M. Clark, S. G. Wong, W. J. Levin, A. Ullrich, and W. L. McGuire. 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science 235:177.*
5. Slamon, D. J., W. Godolphin, L. A. Jones, J. A. Holt, S. G. Wong, D. E. Keith, W. J. Levin, S. G. Stuart, J. Udove, A. Ullrich, et al. 1989. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science 244:707.*
6. Press, M. F., M. C. Pike, V. R. Chazin, G. Hung, J. A. Udove, M. Markowicz, J. Danyluk, W. Godolphin, M. Sliwkowski, R. Akita, et al. 1993. Her-2/neu expression in node-negative breast cancer: direct tissue quantitation by computerized image analysis and association of overexpression with increased risk of recurrent disease. *Cancer Res. 53:4960.*
7. Seshadri, R., F. A. Firgaira, D. J. Horsfall, K. McCaul, V. Setlur, and P. Kitchen. 1993. Clinical significance of HER-2/neu oncogene amplification in primary breast cancer. *J. Clin. Oncol. 11:1936.*
8. Yonemura, Y., I. Ninomiya, A. Yamaguchi, S. Fushida, H. Kimura, S. Ohoyama, I. Miyazaki, Y. Endou, M. Tanaka, and T. Sasaki. 1991. Evaluation of immunoreactivity for erbB-2 protein as a marker of poor short term prognosis in gastric cancer. *Cancer Res. 51:1034.*
9. Berchuck, A., G. Rodriguez, R. B. Kinney, J. T. Soper, R. K. Dodge, D. L. Clarke-Pearson, and R. C. Bast, Jr. 1991. Overexpression of HER-2/neu in endometrial cancer is associated with advanced stage disease. *Am. J. Obstet. Gynecol. 164:15.*
10. Press, M. F., M. C. Pike, G. Hung, J. Y. Zhou, Y. Ma, J. George, J. Dietz-Band, W. James, D. J. Slamon, J. G. Batsakis, et al. 1994. Amplification and overexpression of HER-2/neu in carcinomas of the salivary gland: correlation with poor prognosis. *Cancer Res. 54:5675.*
11. Kallioniemi, O. P., K. Holli, T. Visakorpi, T. Koivula, H. H. Helin, and J. J. Isola. 1991. Association of c-erbB-2 protein over-expression with high rate of cell proliferation, increased risk of visceral metastasis and poor long-term survival in breast cancer. *Int. J. Cancer 49:650.*
12. Pantel, K., G. Schlimok, S. Braun, D. Kutter, F. Lindemann, G. Schaller, I. Funke, J. R. Izbicki, and G. Riethmuller. 1993. Differential expression of proliferation-associated molecules in individual micrometastatic carcinoma cells. *J. Natl. Cancer Inst. 85:1419.*
13. Carter, P., L. Presta, C. M. Gorman, J. B. Ridgway, D. Henner, W. L. Wong, A. M. Rowland, C. Kotts, M. E. Carver, and H. M. Shepard. 1992. Humanization

of an anti-p185HER2 antibody for human cancer therapy. *Proc. Natl. Acad. Sci. USA 89:4285.*
14. Baselga, J., D. Tripathy, J. Mendelsohn, S. Baughman, C. C. Benz, L. Dantis, N. T. Sklarin, A. D. Seidman, C. A. Hudis, J. Moore, et al. 1996. Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. *J. Clin. Oncol. 14:737.*
15. Baselga, J., D. Tripathy, J. Mendelsohn, S. Baughman, C. C. Benz, L. Dantis, N. T. Sklarin, A. D. Seidman, C. A. Hudis, J. Moore, et al. 1999. Phase II study of weekly intravenous trastuzumab (Herceptin) in patients with HER2/neu-overexpressing metastatic breast cancer. *Semin. Oncol. 26:78.*
16. Pegram, M. D., A. Lipton, D. F. Hayes, B. L. Weber, J. M. Baselga, D. Tripathy, D. Baly, S. A. Baughman, T. Twaddell, J. A. Glaspy, et al. 1998. Phase II study of receptor-enhanced chemosensitivity using recombinant humanized anti-p185HER2/neu monoclonal antibody plus cisplatin in patients with HER2/neu-overexpressing metastatic breast cancer refractory to chemotherapy treatment. *J. Clin. Oncol. 16:2659.*
17. Fischer, H. G., S. Frosch, K. Reske, and A. B. Reske-Kunz. 1988. Granulocyte-macrophage colony-stimulating factor activates macrophages derived from bone marrow cultures to synthesis of MHC class II molecules and to augmented antigen presentation function. *J. Immunol. 141:3882.*
18. Heufler, C., F. Koch, and G. Schuler. 1988. Granulocyte/macrophage colony-stimulating factor and interleukin 1 mediate the maturation of murine epidermal Langerhans cells into potent immunostimulatory dendritic cells. *J. Exp. Med. 167:700.*
19. Morrissey, P. J., L. Bressler, L. S. Park, A. Alpert, and S. Gillis. 1987. Granulocyte-macrophage colony-stimulating factor augments the primary antibody response by enhancing the function of antigen-presenting cells. *J. Immunol. 139:1113.*
20. Smith, P. D., C. L. Lamerson, H. L. Wong, L. M. Wahl, and S. M. Wahl. 1990. Granulocyte-macrophage colony-stimulating factor stimulates human monocyte accessory cell function. *J. Immunol. 144:3829.*
21. Blanchard, D. K., and J. Y. Djeu. 1991. Differential modulation of surface antigens on human macrophages by IFN-γ and GM-CSF: effect on susceptibility to LAK lysis. *J. Leukocyte Biol. 50:28.*
22. Steis, R. G., L. A. VanderMolen, D. L. Longo, J. W. Clark, J. W. d. Smith, W. C. Kopp, F. W. Ruscetti, S. P. Creekmore, L. J. Elwood, et al. 1990. Recombinant human granulocyte-macrophage colony-stimulating factor in patients with advanced malignancy: a Phase Ib trial. *J. Natl. Cancer. Inst. 82:697.*
23. Arnaout, M. A., E. A. Wang, S. C. Clark, and C. A. Sieff. 1986. Human recombinant granulocyte-macrophage colony-stimulating factor increases cell-to-cell adhesion and surface expression of adhesion-promoting surface glycoproteins on mature granulocytes. *J. Clin. Invest. 78:597.*
24. Grabstein, K. H., D. L. Urdal, R. J. Tushinski, D. Y. Mochizuki, V. L. Price, M. A. Cantrell, S. Gillis, and P. J. Conlon. 1986. Induction of macrophage tumoricidal activity by granulocyte-macrophage colony-stimulating factor. *Science 232:506.*
25. Young, D. A., L. D. Lowe, and S. C. Clark. 1990. Comparison of the effects of IL-3, granulocyte-macrophage colony-stimulating factor, and macrophage colony-stimulating factor in supporting monocyte differentiation in culture: analysis of macrophage antibody-dependent cellular cytotoxicity. *J. Immunol. 145:607.*
26. Santoli, D., S. C. Clark, B. L. Kreider, P. A. Maslin, and G. Rovera. 1988. Amplification of IL-2-driven T cell proliferation by recombinant human IL-3 and granulocyte-macrophage colony-stimulating factor. *J. Immunol. 141:519.*
27. Dranoff, G., E. Jaffee, A. Lazenby, P. Golumbek, H. Levitsky, K. Brose, V. Jackson, H. Hamada, D. Pardoll, and R. C. Mulligan. 1993. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc. Natl. Acad. Sci. USA 90:3539.*
28. Saito, S., R. Bannerji, B. Gansbacher, F. M. Rosenthal, P. Romanenko, W. D. Heston, W. R. Fair, and E. Gilboa. 1994. Immunotherapy of bladder cancer with cytokine gene-modified tumor vaccines. *Cancer Res. 54:3516.*
29. Vieweg, J., F. M. Rosenthal, R. Bannerji, W. D. Heston, W. R. Fair, B. Gansbacher, and E. Gilboa. 1994. Immunotherapy of prostate cancer in the Dunning rat model: use of cytokine gene modified tumor vaccines. *Cancer Res. 54:1760.*
30. Nagai, E., T. Ogawa, T. Kielian, A. Ikubo, and T. Suzuki. 1998. Irradiated tumor cells adenovirally engineered to secrete granulocyte/macrophage-colony-stimulating factor establish antitumor immunity and eliminate pre-existing tumors in syngeneic mice. *Cancer Immunol. Immunother. 47:72.*
31. Soiffer, R., T. Lynch, M. Mihm, K. Jung, C. Rhuda, J. C. Schmollinger, F. S. Hodi, L. Liebster, P. Lam, S. Mentzer, et al. 1998. Vaccination with irradiated autologous melanoma cells engineered to secrete human granulocyte-macrophage colony-stimulating factor generates potent antitumor immunity in patients with metastatic melanoma. *Proc. Natl. Acad. Sci. USA 95:13141.*
32. Simons, J. W., B. Mikhak, J. F. Chang, A. M. DeMarzo, M. A. Carducci, M. Lim, C. E. Weber, A. A. Baccala, M. A. Goemann, S. M. Clift, et al. 1999. Induction of immunity to prostate cancer antigens: results of a clinical trial of vaccination with irradiated autologous prostate tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor using ex vivo gene transfer. *Cancer Res. 59:5160.*
33. Simons, J. W., E. M. Jaffee, C. E. Weber, H. I. Levitsky, W. G. Nelson, M. A. Carducci, A. J. Lazenby, L. K. Cohen, C. C. Finn, S. M. Clift, et al. 1997. Bioactivity of autologous irradiated renal cell carcinoma vaccines generated by ex vivo granulocyte-macrophage colony-stimulating factor gene transfer. *Cancer Res. 57:1537.*
34. Hrouda, D., M. Perry, and A. G. Dalgleish. 1999. Gene therapy for prostate cancer. *Semin. Oncol. 26:455.*
35. Ruef, C., and D. L. Coleman. 1990. Granulocyte-macrophage colony-stimulating factor: pleiotropic cytokine with potential clinical usefulness. *Rev. Infect. Dis. 12:41.*
36. Zimmer, B. M., W. E. Berdel, W. D. Ludwig, M. Notter, B. Reufi, and E. Thiel. 1993. Fatal spleen rupture during induction chemotherapy with rh GM-CSF priming for acute monocytic leukemia: clinical case report and in vitro studies. *Leuk. Res. 17:277.*
37. Corbett, T. H., D. P. Griswold, Jr., B. J. Roberts, J. C. Peckham, and F. M. Schabel, Jr. 1975. Tumor induction relationships in development of transplantable cancers of the colon in mice for chemotherapy assays, with a note on carcinogen structure. *Cancer Res. 35:2434.*
38. Griswold, D. P., and T. H. Corbett. 1975. A colon tumor model for anticancer agent evaluation. *Cancer 36:2441.*
39. Penichet, M. L., P. M. Challita, S. U. Shin, S. L. Sampogna, J. D. Rosenblatt, and S. L. Morrison. 1999. In vivo properties of three human HER2/neu-expressing murine cell lines in immunocompetent mice. *Lab. Anim. Sci. 49:179.*
40. U.S. Department of Health and Human Services, Public Health Services. 1985. *Guide for the Care and Use of Laboratory Animals.* National Institutes of Health, Bethesda, MD.
41. Challita-Eid, P. M., M. L. Penichet, S. U. Shin, T. Poles, N. Mosammaparast, K. Mahmood, D. J. Slamon, S. L. Morrison, and J. D. Rosenblatt. 1998. A B7.1-antibody fusion protein retains antibody specificity and ability to activate via the T cell costimulatory pathway. *J. Immunol. 160:3419.*
42. Shin, S. U., and S. L. Morrison. 1989. Production and properties of chimeric antibody molecules. *Methods Enzymol. 178:459.*
43. Duerst, R., and K. Werberig. 1991. Cells of the J774 macrophage cell line are primed for antibody-dependent cell-mediated cytotoxicity following exposure to γ-irradiation. *Cell. Immunol. 136:361.*
44. Matzinger, P. 1991. The JAM test: a simple assay for DNA fragmentation and cell death. *J. Immunol. Methods 145:185.*
45. Altman, P. L., and D. D. Katz. 1961. Blood and other body fluids: analysis and compilation by Philip L. Altman. In *Biological Handbooks*. D. S. Dittmer, ed. Federation of American Societies for Experimental Biology, Bethesda, MD.
46. Harvill, E. T., J. M. Fleming, and S. L. Morrison. 1996. In vivo properties of an IgG3-IL-2 fusion protein: a general strategy for immune potentiation. *J. Immunol. 157:3165.*
47. Peng, L. S., M. L. Penichet, and S. L. Morrison. 1999. A single-chain IL-12 IgG3 antibody fusion protein retains antibody specificity and IL-12 bioactivity and demonstrates antitumor activity. *J. Immunol. 163:250.*
48. Dangl, J. L., T. G. Wensel, S. L. Morrison, L. Stryer, L. A. Herzenberg, and V. T. Oi. 1988. Segmental flexibility and complement fixation of genetically engineered chimeric human, rabbit and mouse antibodies. *EMBO J. 7:1989.*
49. Phillips, M. L., M. H. Tao, S. L. Morrison, and V. N. Schumaker. 1994. Human/mouse chimeric monoclonal antibodies with human IgG1, IgG2, IgG3 and IgG4 constant domains: electron microscopic and hydrodynamic characterization. *Mol. Immunol. 31:1201.*
50. Tao, M. H., and S. L. Morrison. 1989. Studies of aglycosylated chimeric mouse-human IgG: role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. *J. Immunol. 143:2595.*
51. Roitt, I. M., J. Brostoff, and K. Male, eds. 1989. *Immunology*, 2nd Ed. Gower Medical Publishing. London.
52. Clynes, R. A., T. L. Towers, L. G. Presta, and J. V. Ravetch. 2000. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nat. Med. 6:443.*
53. Hornick, J. L., L. A. Khawli, P. Hu, M. Lynch, P. M. Anderson, and A. L. Epstein. 1997. Chimeric CLL-1 antibody fusion proteins containing granulocyte-macrophage colony-stimulating factor or interleukin-2 with specificity for B-cell malignancies exhibit enhanced effector functions while retaining tumor targeting. *Blood 89:4437.*
54. Hennemann, B., M. Kreutz, A. Rehm, and R. Andreesen. 1998. Effect of granulocyte-macrophage colony-stimulating factor treatment on phenotype, cytokine release and cytotoxicity of circulating blood monocytes and monocyte-derived macrophages. *Br. J. Haematol. 102:1197.*
55. Penichet, M. L., E. T. Harvill, and S. L. Morrison. 1998. An IgG3-IL-2 fusion protein recognizing a murine B cell lymphoma exhibits effective tumor imaging and antitumor activity. *J. Interferon Cytokine Res. 18:597.*
56. Johnstone, A., and R. Thorpe, eds. 1996. *Immunochemistry in Practice*, 3rd Ed. Blackwell Science, Cambridge, MA, p. 113.
57. Burgess, A. W., and D. Metcalf. 1977. Serum half-life and organ distribution of radiolabeled colony stimulating factor in mice. *Exp. Hematol. 5:456.*
58. Cebon, J. S., R. W. Bury, G. J. Lieschke, and G. Morstyn. 1990. The effects of dose and route of administration on the pharmacokinetics of granulocyte-macrophage colony-stimulating factor. *Eur. J. Cancer 26:1064.*
59. Konrad, M. W., G. Hemstreet, E. M. Hersh, P. W. Mansell, R. Mertelsmann, J. E. Kolitz, and E. C. Bradley. 1990. Pharmacokinetics of recombinant interleukin 2 in humans. *Cancer Res. 50:2009.*
60. Dreier, T., H. N. Lode, R. Xiang, C. S. Dolman, R. A. Reisfeld, and A. S. Kang. 1998. Recombinant immunocytokines targeting the mouse transferrin receptor: construction and biological activities. *Bioconjug. Chem. 9:482.*
61. Dowlati, A., M. Loo, T. Bury, G. Fillet, and Y. Beguin. 1997. Soluble and cell-associated transferrin receptor in lung cancer. *Br. J. Cancer 75:1802.*
62. Chen, T. T., M. H. Tao, and R. Levy. 1994. Idiotype-cytokine fusion proteins as cancer vaccines: relative efficacy of IL-2, IL-4, and granulocyte-macrophage colony-stimulating factor. *J. Immunol. 153:4775.*