Award Number: DAMD17-99-1-9098

TITLE: Antibody-Cytokine Fusion Proteins for the Therapy of Breast Cancer

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REPORT DATE: July 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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Antibody-Cytokine Fusion Proteins for the Therapy of Breast Cancer

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We have proposed to develop novel antibody fusion proteins in which human IgG3 specific for the breast tumor associated antigen HER2/neu will be genetically fused to the cytokines IL-2, IL-12, and GM-CSF. It is expected that the anti-HER2/neu antibody fusion protein will localize the cytokine at the tumor and elicit an immune response. Prior to this second year of funding we had found that these three novel molecules expressed in myeloma cells were correctly assembled and secreted and showed antibody and cytokine related activities. We had also found that anti-HER2/neu IgG3-(IL12) and anti-HER2/neu IgG3-(GM-CSF) exhibited a significant anti-tumor activity. During our second year of funding we conducted studies to define the mechanism of action of anti-HER2/neu IgG3-(IL12). We found that the anti-tumor activity exhibited by anti-HER2/neu IgG3-(IL12) is highly complex and involves a combination of T and NK cell activity, a switch to a Th1 immune response and anti-angiogenic activity. We also completed the initial in vivo study of anti-HER2/neu IgG3-(IL2). We found that treatment of immunocompetent mice with anti-HER2/neu IgG3-(IL2) resulted in significant retardation in the subcutaneous growth of CT26-HER2/neu tumors. Our results suggest that our proposed antibody fusion proteins will be useful for the treatment of human cancer.

Immunology, immunotherapy, antibody fusion protein, cytokine

Unlimited
INTRODUCTION

In the present grant we propose to explore the use of genetically engineered antibodies as therapeutic agents specifically attempting to augment and potentiate the host immune defense systems against breast cancer. We will use antibodies specific for HER2/neu, a molecule present on the surface of many breast cancers; its increased expression is associated with poor prognosis. To this antibody we will join the cytokines IL-2, IL-12, and GM-CSF. Expression of these cytokines by cancer cells has been shown to render them immunogenic. The anti-HER2/neu will be used to localize the cytokine at the tumor where it is expected to elicit an immune response. The resulting immune response would be expected to be specific not only for the targeting antigen, but also for other tumor associated antigens resulting in the destruction of both the tumor cells which express the targeting antigen as well as those that do not. Simultaneous targeting of more than one cytokine to the tumor would be expected to lead to synergism in immune activation and an even more potent immune response.
During our first year of funding we completed the construction, expression and characterization (in vitro and in vivo) of an anti-HER2/neu IgG3-(GM-CSF) fusion protein. By the time of our first annual report we had submitted a manuscript entitled “A recombinant anti-human HER2/neu IgG3-(GM-CSF) fusion protein retains antigen specificity, cytokine function and demonstrates anti-tumor activity” by Jay S. Dela Cruz, K. Ryan Trinh, Sherie L. Morrison, and Manuel L. Penichet. It described in detail the in vitro and in vivo properties of this novel molecule and was attached as an appendix in our first annual report. During the second year of funding the manuscript was revised and published in Journal of Immunology (1). Similarly, during the first year of funding we submitted a manuscript entitled “A murine B cell lymphoma expressing human HER2/neu undergoes spontaneous tumor regression and elicits anti-tumor immunity” by Manuel L. Penichet, Jay S. Dela Cruz, Pia M. Challita-Eid, Joseph D. Rosenblatt and Sherie L. Morrison. It described in detail the in vitro and in vivo properties of a tumor model (38C13-HER2/neu) and was attached as an appendix in our first annual report. During the second year of funding the manuscript was revised and published in Cancer Immunology and Immunotherapy (2).

Prior to the funding of this proposal we had completed the initial characterization of anti-HER2/neu IgG3-(IL-12) fusion proteins (3). The publication describing these initial studies was attached as an appendix in our first annual report. Studies to define the mechanism of action of anti-HER2/neu IgG3-(IL-12) were conducted during this second year of funding. We found that the anti-tumor activity of antibody-(IL-12) is dose-dependent and comparable or better than recombinant IL-12 using subcutaneous and metastatic models of disease. The anti-tumor activity of anti-HER2/neu IgG3-(IL-12) is reduced in Rag2 knockout mice, suggesting that T cells play a role in tumor rejection. In SCID-beige mice, the anti-tumor activity is further reduced, suggesting that NK cells and/or macrophages are also important. The isotype of the antibody response to HER2/neu is consistent with a switch from a Th2 to a Th1 immune response and the infiltration of mononuclear cells is seen in tumors from mice treated with anti-HER2/neu IgG3-(IL-12). Immunohistochemistry reveals that anti-HER2/neu IgG3-(IL-12) is anti-angiogenic. Thus, the mechanism of the anti-tumor activity exhibited by anti-HER2/neu IgG3-(IL-12) is highly complex and involves a combination of T and NK cell activity, a switch to a Th1 immune response and anti-angiogenic activity. This is the first study comparing the in vivo anti-tumor activity of an antibody-(IL-12) fusion protein and free IL-12. Our results are described in a paper entitled “Mechanism of antitumor activity of a single-chain IL-12 IgG3 antibody fusion protein (mscIL-12.her2.1gG3)” by Peng L, Penichet M.L., Dela Cruz J.S., Sampogna S.L., and Morrison S.L. which is in press in Journal of Interferon and Cytokine Research (4).

We also completed the initial in vivo study of anti-HER2/neu IgG3-(IL-2). We found that treatment of immunocompetent mice with this antibody fusion protein resulted in significant retardation in the subcutaneous growth of CT26-HER2/neu tumors under conditions in which the antibody alone fails to confer protection, suggesting that anti-HER2/neu IgG3-(IL-2) fusion protein will be useful in the treatment of HER2/neu expressing tumors. We also found that fusing IL-2 to human IgG3 results in a significant enhancement of the murine anti-human antibody (MAHA) response. Our initial results are described in a paper entitled “A recombinant IgG3-

KEY RESEARCH ACCOMPLISHMENTS

1) The definition of the mechanism of action of anti-HER2/neu IgG3-(IL-12) fusion proteins. These are the first studies addressing the mechanism of anti-tumor activity of an anti-HER2/neu IgG3-(IL-12) fusion protein.

2) The construction, expression and initial in vivo characterization of anti-human HER2/neu IgG3-(IL-2) fusion protein.

REPORTABLE OUTCOMES

Manuscripts:

Two original research manuscripts were submitted by the time of the first annual report. Both were accepted and published during the second year of funding:


Two original research manuscripts, one review manuscript, and one book chapter were submitted and accepted for publication by the time of this second annual report:


Presentations and Abstracts:


In all our papers and meeting presentations we included the phrase: “This work was supported by Department of Defense Breast Cancer Research Program Grant BC980134.”

CONCLUSIONS

Our results suggest that the three antibody fusion proteins we have developed: anti-HER2/neu IgG3-(GM-CSF), anti-HER2/neu IgG3-(II2), and anti-HER2/neu IgG3-(IL12) may be effective in patients with tumors overexpressing HER2/neu. The combination of an anti-HER2/neu antibody with GM-CSF, IL-2 or IL-12 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 anti-tumor immune response through the immunostimulatory activity of GM-CSF, IL-2 or IL-12. In the specific case of anti-HER2/neu IgG3-(IL12) the anti-angiogenic activity of IL-2 also contributes to the anti-tumor activity of this antibody fusion protein. In addition, the anti-HER2/neu antibody fusion protein may be effective against tumor cells which express a truncated form of ECDHER2 lacking the receptor function rendering them particularly resistant to anti-HER2/neu antibody therapy. Because of the cytokine’s (GM-CSF, IL-2, and IL-12) ability to elicit an immune response to associated antigens, it is also possible that association of anti-HER2/neu IgG3-cytokine fusion proteins with soluble ECDHER2 shed by tumor cells will enhance the anti-tumor immune response.

More studies are required to define the optimal dose and injection schedule for our antibody fusion proteins, continue the study of mechanism of their anti-tumor activity, the potential side effects, and to explore it is effectiveness against other human HER2/neu expressing murine tumor models.

REFERENCES


APPENDICES

The reprints of the paper listed in section 10 (REFERENCES), and the original manuscript No. 4 which is still in press are included.
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 (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) 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 (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 1 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 Abbreviations used in this paper: TAA, tumor-associated Ag; DNS, N,N-dimethyl-
1-aminobiphenylene-5-sulfon) chloride (dansyl); mGM-CSF, recombinant murine GM-CSF; ECDHER2, extracellular domain of HER2/neu Ag; AP, alkaline phospha-
tase; ADCC, Ab-dependent cellular cytotoxicity; %ID/g tissue, percent of injected
dose per gram of tissue.

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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 TAAa 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 immuno-competent 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 automated 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-C2×3-(GM-CSF)

The DNA encoding the variable light (Vλ) and heavy (VH) chain domains of the humanized Ab huMAB-HER2 (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'-CCCTGCGGGCGGAAACCCACCGGTCCACCC-3' and the antisense primer 5'-CCGAATTCGGTTTAGGCATCTTTTTGAGCTGTTTTGAGAC-3'.

The PCR product was digested with NotI/EcoRI and cloned in the vector pTA3462 (previously developed in our laboratory) digested with SphI/EcoRI, yielding the vector pTA7191 (Fig. 1). The plasmids pTA6611, pAH4874 (both previously developed in our laboratory), and pTA7191 were digested with EcoRV/NsiI, EcoRV/BamHI, and NsiI/BamHI, respectively. The fragments containing the DNA encoding for anti-HER2/neu VH and γ5 constant regions (from pTA6611), the expression vector backbone (from pAH4874), and GM-CSF (from pTA7191) 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-C2×3-(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 mycoplasma 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 [35S]methionine (ICN, Irvine, CA), and the fusion protein was immunoprecipitated using rabbit anti-human IgG and a 10% suspension of staphylococcal protein A (IgG Sorb, 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-agarose 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 Coomasie 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 (105) 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 IgG Abs were used as positive and negative isotype-matched control reagents, respectively. Cells were washed and resuspended 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 Coomasie 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.

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 ED50 ≤ 0.2 ng/ml (Chemicon, Temecula, CA) reconstituted using deionized water following the manufacturer’s recommen- dations 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, 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% CO2, 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 Duerr 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 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 × 10−5 μg/ml anti-HER2/neu IgG3-(GM-CSF) (equivalent to 50
ANTI-HUMAN HER2/neu IgG3-(GM-CSF) FUSION PROTEIN

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 diosodium 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 naïve 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 x (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-C123-(GM-CSF)**

The strategy for the construction and expression of anti-HER2/neu IgG3-C123-(GM-CSF) is illustrated in Fig. 1. Clones expressing anti-HER2/neu IgG3-C123-(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-C123-(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. 2A). 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 H₂L₂ 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

**Half-life**

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 time intervals, 125I-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) 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 percent of injected dose per gram of tissue (%ID/g tissue). Values were corrected for the radioactivity in blood and 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 (10⁵ 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 × 10⁶ 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<sup>445-448</sup> (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
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 rGM-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 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 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) 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 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

**FIGURE 5.** Macrophage-mediated cytotoxicity. A. A total of 1 × 10⁸ ³H-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 × 10⁻⁷ µg/ml), the equivalent molar concentration of anti-HER2/neu IgG3, or nothing; washed; and then incubated with 1 × 10⁸ ³H-labeled CT26-HER2/neu target cells for 24 h. For both assays, intact DNA figure live target cells was collected by a cell harvester, and radioactivity was measured using a scintillation counter. Bars represent the SD of quadruplicate samples.
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 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, 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).

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**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.

**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.
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 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 C4d3 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/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...
using anti-HER2/new 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/new IgG3-(GM-CSF) is rapid compared with anti-HER2/new 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/new 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/new 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/new 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/new 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/new IgG3-(GM-CSF) causes a significant retardation in the growth of s.c. CT26-HER2/new tumors under conditions in which anti-HER2/new 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/new 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

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Groups of eight mice injected s.c. with 10⁶ CT26-HER2/new cells were treated beginning the next day with five daily i.v. injections of 0.25 ml PBS containing 20 μg anti-HER2/new IgG3-(GM-CSF), the equivalent molar amount of anti-HER2/new 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 ECTWIR8 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.

*Groups of eight mice injected s.c. with 10⁶ CT26-HER2/new cells were treated beginning the next day with five daily i.v. injections of 0.25 ml PBS containing 20 μg anti-HER2/new IgG3-(GM-CSF), the equivalent molar amount of anti-HER2/new 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 ECTWIR8 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 ADCP 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 ECDHER2 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 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 hamAb4D5 Ab, Dr. Matthew Scharff for the cell line J774.2, Dr. James D. Marks for ECDHER2, and Dr. Donald Morrison for assistance with statistical analysis.

References


A murine B cell lymphoma expressing human HER2/neu undergoes spontaneous tumor regression and elicits antitumor immunity

Received: 2 August 2000 / Accepted: 20 September 2000

Abstract In the present study we describe a novel murine tumor model in which the highly malignant murine B cell lymphoma 38C13 has been transduced with the cDNA encoding human tumor-associated antigen HER2/neu. This new cell line (38C13-HER2/neu) showed stable surface expression but not secretion of human HER2/neu. It also maintained expression of the idiotypic (Id) of the surface immunoglobulin of 38C13, which serves as another tumor-associated antigen. Surprisingly, spontaneous tumor regression was observed following s.c. but not i.v. injection of 38C13-HER2/neu cells in immunocompetent syngeneic mice. Regression was more frequently observed with larger tumor cell challenges and was mediated through immunological mechanisms because it was not observed in syngeneic immunodeficient mice. Mice that showed complete tumor regression were immune to challenge with the parental cell line 38C13 and V1, a variant of 38C13 that does not express the Id. Immunity could be transferred with sera, suggesting that an antibody response mediated rejection and immunity. Continuously growing s.c. tumors as well as metastatic tumors obtained after the i.v. injection of 38C13-HER2/neu maintained expression of human HER2/neu, which can serve as a target for active immunotherapy. As spontaneous tumor regression has not been observed in other human murine models expressing human HER2/neu, our results illustrate the enormous differences that can exist among different murine tumors expressing the same antigen. The present model provides a useful tool for the study of the mechanisms of protective immunity to B cell lymphoma and for the evaluation of different therapeutic approaches based on the stimulation or suppression of the immune response.

Key words Tumor model · HER2/neu · Xenogenization · Immunotherapy · Lymphoma

Introduction

The HER2/neu proto-oncogene (also known as c-erbB-2) encodes a 185-kDa transmembrane glycoprotein receptor that has partial homology with the epidermal growth factor receptor and shares with that receptor intrinsic tyrosine kinase activity. It consists of three domains: a cysteine-rich extracellular domain, a transmembrane domain and a short cytoplasmic domain [1, 12, 39]. HER2/neu is expressed at low levels on some normal cells; however, markedly increased expression has been observed in many human breast, gastrointestinal, lung and ovarian cancers [16, 35–37, 42]. The elevated levels of the HER2/neu protein in malignancies and the extracellular accessibility of this molecule make it an excellent candidate for tumor-specific therapeutic agents. In fact, treatment of patients with advanced breast cancer using the anti-HER2/neu antibody, trastuzumab (Herceptin, Genentech, San Francisco, Calif.), leads to an objective response in a subset of patients with tumors overexpressing the HER2/neu oncoprotein [2, 3, 45]. These results justify recent enthusiasm for continued efforts to refine existing approaches and to develop new strategies that target HER2/neu.

We have developed a family of anti-(human HER2/neu) antibody fusion proteins containing immunostim-
ulatory molecules such as the such as the cytokine interleukin-12 (IL-12) [29], costimulatory molecules such as B7.1 [10] or chemokines such as RANTES [11]. To evaluate the immunological efficacy of these proteins, it is critical that tumors expressing the target antigen can grow in immunologically competent mice. To produce murine tumors expressing human HER2/neu, we transduced the murine colon adenocarcinoma cell lines CT26 and MC38 and the murine T cell lymphoma EL4 with the cDNA encoding the human HER2/neu. We showed that those cells were able to grow in immunocompetent mice while maintaining the expression of human HER2/neu [31] and such models are now being used for preclinical evaluation of the efficacy of anti-HER2/neu antibody fusion proteins [29] (M.L. Penichet et al. unpublished results).

To further expand our repertoire of human-HER2/neu-expressing murine tumors, we have developed a new model using the highly malignant murine B cell lymphoma 38C13. Although overexpression of HER2/neu has been mainly associated with breast, gastrointestinal, lung and ovarian cancers [16, 35–37, 42], it has also been described for B cell lymphomas [17, 18]. Thus, 38C13 expressing human HER2/neu may be used to evaluate the efficacy of antibody fusion proteins against B cell lymphoma. If these proteins are effective they can be further evaluated in clinical trials targeting lymphomas expressing HER2/neu or they can be modified to target other tumor-associated antigens (TAA) found on B cell lymphomas. In addition, since the Id of the surface immunoglobulin of 38C13 has been previously used to target anti-Id antibody fusion proteins [25, 30], the 38C13-HER2/neu model will allow us to test the potential synergistic effect of different antibody fusion proteins targeting two different TAA: the Id and HER2/neu. Moreover, the availability of different cell lines expressing the same antigen allows us to test the efficacy of HER2/neu-targeted approaches in a variety of cell lines and/or mouse strains and it is well known that different responses to the same anticancer therapy are exhibited by different tumors [30].

In this report, we describe the transduction of 38C13 with a retroviral construct containing the full-length cDNA encoding the human HER2/neu gene. These cells (38C13-HER2/neu) show stable expression of human HER2/neu on their surface while maintaining expression of the surface immunoglobulin. The biological properties of this transduced cell line were analyzed after transplantation into immunologically intact syngeneic mice. Parameters that were investigated include tumor growth rate and phenotype, ability to produce metastases, expression of HER2/neu, antigen shedding and the anti-(human HER2/neu) response of the host. Contrary to our expectations, spontaneous tumor regression after temporary growth was observed following s.c. injection of tumor cells, and this regression was more frequently observed the greater the number of cells used to elicit tumor growth; however, regression was not observed following i.v. injection of tumor cells.

Materials and methods

Cells

38C13 is a C3H/HeN murine B cell lymphoma expressing a surface μκ antibody (Id) that arose in a carcinogen(7,12-dimethylbenz[a]anthracene)-treated mouse [4, 5]. V1 is an Id-negative variant derived from the original 38C13 tumor [38]. Both cell lines were kindly provided by Drs. Ronald and Shoshana Levy (Stanford University, Stanford, Calif.). The parental and transduced cells were maintained in Iscove’s modification of Dulbecco’s medium (Irvine Scientific Inc., Irvine, Calif.) containing 10% calf serum supplemented with iron (Atlanta Biologicals, Norcross, Ga.) at 37°C with 5% CO₂.

Mice

Female immunocompetent C3H/HeN mice and Rag2 double-knockout mice lacking mature T and B cell lymphocytes with the C3H/HeN background, between 6–8 weeks of age, obtained from Taconic Farms Inc. (Germantown, N.Y.) were used. The mice received food and water ad libitum. Artificial light was provided under a 12 h/12 h light/dark cycle. The temperature of the facility was 20°C with 10–15 air exchanges/h. All experiments were performed according to National Institutes of Health (NIH) (Bethesda, Md.) Guide for the care and use of laboratory animals.

Retroviral expression vector, transduction and screening

The cDNA for HER2/neu cloned in retroviral vector based on a Moloney murine leukemia virus (MoMuLV), including the neomycin-resistance gene (neo) under the control of the SV40 promoter, was used for transduction of 38C13. This vector was previously used to derive other human HER2/neu-expressing murine cell lines [31]. Cells were selected with Geneticin (Sigma Chemical, St. Louis, Mo.). HER2/neu expression on the surface of transduced cells was detected with an immunofluorescence assay. Samples of 10⁶ cells were incubated with 1 μg recombinant anti-HER2/neu antibody for 2 h at 4°C. Recombinant anti-Id and recombinant anti-DNS (DNS is the hapten N,N-dimethyl-l-aminonaphthalene-5-sulfonyl chloride, also known as dansyl) antibodies were used as positive and negative isotype-matched controls respectively. All three recombinant antibodies were developed in our laboratory and contained human κ and γ3 constant regions. Cells were washed and, following incubation for 2 h at 4°C with biotinylated goat anti-(human IgG) (Pharmingen, San Diego, Calif.), were washed and incubated for 30 min with phycoerythrin-labeled streptavidin (Pharmingen, San Diego, CA). Analysis was performed with a FACScan flow cytometer (Becton-Dickinson, Mountain View, Calif.) equipped with a blue laser excitation of 15 mW at 488 nm.

Determination of the presence of extracellular domain of HER2/neu antigen (ECDHER2) in cell culture supernatant

ECDHER2 was detected by enzyme-linked immunosorabsorbent assay (ELISA). Ninety-six well microtiter plates were coated with 50 μl recombinant human anti-HER2/neu IgG3 (developed in our laboratory) at a concentration of 1 μg/ml. The plates were blocked with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and dilutions of cell culture supernatant or ECDHER2 (kindly provided by Dr. James D. Marks, UCSF, San Francisco, Calif.) in PBS containing 1% BSA were added to the wells and incubated overnight at 4°C. The wells were then washed with PBS and incubated for 2 h at room temperature with the anti-HER2/neu mAb Neu 9C6 (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.). After washing with PBS, alkaline-phosphatase-labeled goat anti-mouse IgG (Sigma Chemical, St. Louis, Mo.) was added and the
plates were incubated for 1 h at 37 °C. After washing, disodium p-nitrophenyl phosphate dissolved in diethanolamine buffer (Sigma Chemical, St. Louis, Mo.) was added to the wells for 1 h and plates were read at 410 nm.

s.c. and i.v. transplantation of tumor cell lines

Mice were injected s.c. in the right flank with 38C13-HER2/neu in 0.15 ml Hank’s balanced salt solution (HBSS) (Gibco BRL, Grand Island, N.Y.). In order to compare the s.c. growth of the transduced cell lines with that of their respective parental cell line, additional mice were injected with the same dose of 38C13. Tumor growth was measured three times a week with a caliper and the length of survival recorded. Mice were injected i.v. with 38C13-HER2/neu or with the corresponding dose of 38C13 in 0.3 ml HBSS (Gibco BRL, Grand Island, N.Y.) via the lateral tail vein, and the length of survival was recorded.

Histological study

Mice were injected s.c. with 38C13-HER2/neu or 38C13 and tumor growth monitored described above. Mice were humanely killed 24 h after tumor regression and a histological study of their respective tumors was carried out on paraffin-embedded tissue samples. Sections of 6 μm were stained with hematoxylin and eosin.

Passive transfer of sera and splenocytes

A pool of sera and splenocytes was prepared from mice that had shown tumor regression and survived 60 days without new evidence of tumor following injection with 38C13-HER2/neu. According to previous reports, mice inoculated with 38C13 are free of tumor 60 days after the injection are, in fact, cured of lymphoma [6, 7, 30]. A splenocyte suspension was prepared by mincing and compressing freshly resected spleens between two slides on a petri dish in the presence of HBSS. The splenic capsule was discarded and the cell suspension was transferred to a polystyrene tube. After washing twice with HBSS, the cells were counted using crystal violet staining (Sigma Chemical, St. Louis, Mo.). To transfer immunity using splenocytes, 5 × 10⁶ cells in 0.3 ml HBSS were injected into the tail vein of naïve syngeneic recipient mice. All mice receiving splenocytes were injected i.p. with 100 IU heparin (Sigma Chemical, St. Louis, Mo.) 30 min before the transfer of the splenocytes. To transfer immunity using sera, 0.3 ml pooled sera was injected into the tail vein of naïve syngeneic recipient mice. One day later, mice were challenged s.c. with a lethal dose (10⁶) of the parental tumor 38C13 cells. Tumor incidence and survival were monitored. Naïve syngeneic recipient mice that did not receive splenocytes or sera, or that received splenocytes or sera from naïve mice of a similar age to the long-term survivors, were used as controls.

Determination of murine anti-HER2/neu and anti-Id antibodies

The presence of antibodies to human HER2/neu or to murine Id in mice sera was determined by ELISA using 96-well microtiter plates coated with 50 μl (at a concentration 1 μg/ml) of ECDHER2 or with Id obtained from concentrated supernatant of the hybridoma A1-2, which secretes high levels of soluble 38C13 Id [28]. 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. The wells were then washed with PBS, alkaline-phosphatase-conjugated goat anti-mouse IgG (Sigma Chemical, St. Louis, Mo.) was added, and the plates were processed as described above. As a negative control for determining anti-HER2/neu titers, we used sera from mice of the same age bearing tumors of non-transduced 38C13. As a positive control for determining anti-Id titers we used sera from naïve mice of the same age. All ELISA for comparison of titers between 38C13 and 38C13-HER2/neu were carried out simultaneously in duplicate and using an internal positive control curve for each plate.

Detection of HER2/neu and Id expression in tumors by flow cytometry

Single-cell suspensions from 38C13-HER2/neu and 38C13 were prepared by mincing and pipetting freshly isolated tumors in cold medium. The detection of Id and HER2/neu surface expression on a fresh single-cell suspension, as well as on the same cells kept for 1 week in tissue culture, was done by flow cytometry, as described above.

Statistical analysis

Statistical analysis of the differential findings between experimental groups of mice was done using the nonparametric Wilcoxon-Mann-Whitney rank-sum test. This allows us to include both dead mice as well as the long-term survivors in the analysis.

Results

In vitro human HER2/neu expression in transduced cells

The murine tumor cell line 38C13 was transduced with the retroviral vector containing the HER2/neu cDNA under the control of the MoMuLV enhancer/promoter (38C13-HER2/neu). A stable pool of cells selected in genetin was tested for surface expression of human HER2/neu by flow cytometry. Human HER2/neu expression was detected on the surface of the transduced cell line (Fig. 1B). Incubation of the anti-HER2/neu IgG3 with 38C13-HER2/neu in the presence of excess soluble ECDHER2 abrogated binding (data not shown), confirming the specificity of the recombinant anti-HER2/neu IgG3 used in this assay. The expression of the Id of the pk surface immunoglobulin was also detected by flow cytometry (Fig. 1C). The level of Id expression in 38C13-HER2/neu was similar to the level found on the parental cell line (Fig. 1F).

The above results confirmed the surface expression of human HER2/neu. Secretion of the ECDHER2 has been reported for some HER2/neu-expressing tumors [24, 32]. To address this issue, we quantified the amount of ECDHER2 present in the culture supernatant of 38C13-HER2/neu cells grown at 10⁶/ml and incubated for 24 h. Although the ELISA assay used can detect more than 2 ng/ml soluble recombinant ECDHER2, we did not detect the presence of the ECDHER2 in culture supernatants of cells carried in tissue culture or isolated from tumors and expanded in vitro (data not shown).

s.c. tumor growth characteristics

The growth kinetics in the s.c. space of normal syngeneic mice of 38C13-HER2/neu was compared to that of the parental cell line 38C13. Doses of 10⁴ and 10⁵ 38C13 cells injected s.c. have been shown to yield tumors in 100% of mice [6, 7, 20, 25, 28, 30]. For this reason, we
injected groups of 5 mice in the right flank with $10^3$, $10^4$, $10^5$ or $10^6$ 38C13-HER2/neu or 38C13 cells. 100% of the mice developed tumors with a similar time of tumor onset for each dose of 38C13-HER2/neu and 38C13 (Fig. 2). As expected, higher doses of both cell lines resulted in shorter latency. However, when the tumors reached a size of approximately 1 cm in diameter, some of the 38C13-HER2/neu tumors showed spontaneous regression. This phenomenon appeared to be dose-related as complete regression was more frequently observed with larger tumor cell challenges: 1/5 for $10^3$, (20%), 4/5 for $10^4$ (80%), 4/5 for $10^5$ (80%) and 5/5 for $10^6$ (100%) (Fig. 3, Table 1). All mice showing complete tumor regression became long-term survivors. As expected, all mice injected with $10^3$, $10^4$ or $10^5$ 38C13 cells developed progressive tumors and died (Figs. 2, 3). Surprisingly, 1 of the 5 mice injected with $10^6$ 38C13 cells also showed spontaneous tumor rejection and became a long-term survivor. The survival of mice inoculated with $10^6$, $10^5$ and $10^6$ 38C13-HER2/neu cells was significantly better than that of mice injected with 38C13 ($P < 0.05$). Although the survival of mice injected with $10^5$ cells was not significantly different whether 38C13-HER2/neu or 38C13 was used, 1 of 5 mice injected with 38C13-HER2/neu became a long-term survivor, while all of the mice injected with 38C13 cells died. Post-mortem studies of mice injected with s.c. 38C13 or 38C13-HER2/neu tumors revealed the presence of metastatic tumors in lymph nodes throughout the body (data not shown).

A repetition of this experiment using 10 mice/group for injections of $10^3$, $10^4$ or $10^5$ cells and 20 mice/group for injection of $10^6$ cells gave similar results (Table 1). Larger tumor cell challenges of 38C13-HER2/neu were associated with more frequent complete spontaneous tumor regression. As expected, the injection of $10^3$, $10^4$ or $10^5$ 38C13 cells resulted in progressively growing tumors in 100% of mice, but following the injection of $10^6$ 38C13, spontaneous tumor regression was seen in 2 of 20 (10%) mice.

Two months after the s.c. tumors regressed, long-term survivors were challenged with the $10^4$ 38C13 cells in the left flank. At this tumor cell dose all naive mice of the same age showed progressive tumor growth and died. In contrast, 100% of the long-term survivors previously injected with $10^3$, $10^4$ or $10^5$ 38C13-HER2/neu remained free of tumor (Table 2). Similar results were observed with 2 of the 3 (67%) mice previously injected with $10^4$ 38C13-HER2/neu, suggesting that this lower dose is not only associated with less frequent spontaneous tumor regression but also with weaker antitumor
Fig. 2 Kinetics of tumor growth. Groups of 5 mice were injected in the right flank with $10^3$, $10^4$, $10^5$ or $10^6$ cells from either 38C13 or 38C13-HER2/neu. Tumor growth was monitored and measurements were recorded three times per week with a caliper.

Table 1 Survival of C3H/HeN mice challenged with different doses of 38C13 or 38C13-HER2/neu

<table>
<thead>
<tr>
<th>Tumor injection</th>
<th>Number of mice</th>
<th>Number of survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^3$ 38C13-HER2/neu</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>$10^4$ 38C13-HER2/neu</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>$10^5$ 38C13-HER2/neu</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>$10^6$ 38C13-HER2/neu</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>$10^3$ 38C13</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>$10^4$ 38C13</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>$10^5$ 38C13</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>$10^6$ 38C13</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^3$ 38C13-HER2/neu</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>$10^4$ 38C13-HER2/neu</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>$10^5$ 38C13-HER2/neu</td>
<td>10</td>
<td>4</td>
</tr>
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<td>0</td>
</tr>
<tr>
<td>$10^6$ 38C13</td>
<td>20</td>
<td>2</td>
</tr>
</tbody>
</table>

Fig. 3A, B Survival of C3H/HeN mice (5/group) inoculated s.c. with $10^3$ (○), $10^4$ (●), $10^5$ (□), or $10^6$ (■) 38C13 (A) or 38C13-HER2/neu (B).

immunity. When long-term survivors previously injected with $10^6$ 38C13-HER2/neu were challenged with a lethal dose of $10^4$ V1 cells, an Id-negative variant derived from the original 38C13, 100% of mice remained free of
Table 2 Survival of mice challenged with 38C13 or V1 cells. C3H/HeN mice that showed complete tumor rejection following injection with 38C13-HER2/neu were challenged with a lethal dose (10^6) of 38C13 or V1, an Id-negative variant derived from 38C13. The challenge was made 2 months after the primary tumor had completely regressed. As controls, the same number of naïve C3H/HeN mice of similar age were injected with 38C13 or V1.

<table>
<thead>
<tr>
<th>Previous dose of injection</th>
<th>Challenge</th>
<th>Number of mice</th>
<th>Number of survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^3</td>
<td>38C13 (10^6)</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>10^4</td>
<td>38C13 (10^6)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>10^5</td>
<td>38C13 (10^6)</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>10^6</td>
<td>38C13 (10^6)</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>None (controls)</td>
<td>38C13 (10^6)</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td>10^6</td>
<td>V1 (10^6)</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>None (controls)</td>
<td>V1 (10^6)</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

Tumor while all naïve mice of the same age showed progressive tumor growth and died (Table 2).

To compare the histology of regressing and non-regressing tumors, groups of 5 mice were injected s.c. in the right flank with 10^6 38C13-HER2/neu or 38C13. As expected, all of the mice initially developed tumors (data not shown). By day 11 after the injection of the cells, regression had begun in all of the mice injected with 10^6 38C13-HER2/neu. No tumor regression was observed in mice injected with 10^6 38C13. Figure 4 shows histological sections stained with hematoxylin/eosin of tumors obtained from mice 12 days following injection. The 38C13 tumor is a highly cellular tumor that infiltrates the subcutaneous space and part of the muscular coat (Fig. 4A). Examination of regressing 38C13-HER2/neu tumor was characterized by intense eosinophilia, cell shrinkage, loss of structure and fragmentation, all classic images of necrosis [26] (Fig. 4B). Similar results were observed in all mice injected with 38C13-HER2/neu cells (data not shown). All mice injected with 38C13 had highly cellular tumors similar to the tumor depicted in Fig. 4A.

To test whether tumor regression was immunologically mediated, groups of 8 syngeneic Rag2 double-knockout mice, which lack mature T and B lymphocytes, received s.c. injections of 10^6 38C13 or 38C13-HER2/neu in the right flank. In contrast to what had been observed with immunocompetent mice, both cell lines showed similar tumor growth and no tumor regression was observed in mice injected with 38C13 or 38C13-HER2/neu (data not shown).

Passive transfer of sera and splenocytes from long-term survivors

To determine if humoral or cellular immunity was responsible for the protection observed in mice showing spontaneous tumor regression, either serum or splenocytes from mice that had previously rejected s.c. 38C13-HER2/neu tumors was transferred to naïve C3H/HeN mice 24 h prior to tumor challenge. Transfer of splenocytes or serum was found to confer significant protection to challenge 24 h later, with 10^4 38C13 cells causing either retardation or resistance to tumor growth (Fig. 5). Survival of mice receiving splenocytes or serum from immune mice was significantly greater than that of the control group that did not receive treatment: P < 0.001 for mice receiving splenocytes and P = 0.01 for mice receiving serum. Mice receiving a similar amount of serum or splenocytes from naïve mice showed survival curves similar to those of mice that did not receive any treatment (data not shown).

i.v. tumor growth characteristics

The in vivo studies described above have been restricted to tumors growing in the s.c. space. However, as the route of injection can influence the growth potential of certain cell lines, we also investigated the growth of 38C13-HER2/neu after i.v. injection. Groups of 5 mice were injected i.v. with 10^3, 10^4, 10^5, or 10^6 38C13-HER2/neu or 38C13 cells. We found that, after injecting the cells i.v., all of the mice injected with 38C13-HER2/neu or 38C13 developed a disseminated malignant disease leading to death (Fig. 6). No long-term survivors were observed. Post-mortem studies revealed the presence of tumor metastases in the lymph nodes throughout the body in all mice. Similar results were obtained when this experiment was repeated (data not shown).

Determination of the anti-(human HER2/neu) and anti-Id response in mice bearing s.c. and i.v. tumors

To determine if anti-(human HER2/neu) or anti-Id antibodies were elicited in mice bearing s.c. tumors, groups of 5 mice inoculated s.c. with 10^4 38C13-HER2/neu or 38C13 tumor cells were bled every 3 days for 15 days following injection of the cells. Table 3 shows that anti-(human HER2/neu) antibodies were seen in all of the mice bearing HER2/neu expressing tumors by day 12 with response detectable in 2/5 (40%) of the mice 6 days after challenge. The sera were also tested by ELISA for the presence of anti-Id (Table 3). We did not detect anti-Id in mice bearing the parental tumor 38C13. However, in mice bearing 38C13-HER2/neu tumors a detectable anti-Id response was observed in 3/5 (60%) by day 12. This anti-Id immune response was observed in 3 of the 4 mice showing tumor regression but not in the mouse with a continuously growing 38C13-HER2/neu tumor.

To compare the titers of anti-(human HER2/neu) or anti-Id antibodies elicited in mice bearing s.c. tumors induced by different doses of cells, groups of 5 mice (randomly selected) injected in the right flank with 10^3, 10^4, 10^5 or 10^6 38C13-HER2/neu or 38C13 cells were bled 12 days after the injection of the cells and the sera analyzed for the presence of anti-(human HER2/neu) or anti-Id antibodies (Table 4). No anti-human HER2/neu or anti-Id was detected in sera collected from mice injected with 10^3 38C13-HER2/neu. However, at higher doses of 38C13-HER2/neu cells, both anti-(human HER2/neu) and anti-Id responses were confirmed.
and anti-Id antibodies were seen. Consistent with the results presented in Table 3, mice injected with 10^4 38C13 did not show an anti-Id response by day 12 while 3/5 of mice injected with 10^5 38C13-HER2/neu showed anti-Id titers. Inoculation with higher doses (10^5 or 10^6) of 38C13-HER2/neu resulted in higher anti-Id titers than when an equivalent dose of 38C13 was used. It is important to note that no direct correlation is seen between the magnitude of the anti-(human HER2/neu) response and the survival of mice, suggesting that the anti-(human HER2/neu) antibody response may not significantly contribute to survival. In contrast, the anti-Id response
Table 3 Kinetics of anti-(human HER2/neu) and anti-Id antibody response in mice bearing 38C13-HER2/neu or 38C13 tumors. Groups of 5 mice were injected in the right flank with 10⁴ 38C13-HER2/neu or 38C13 cells. Mice were bled every 3 days and the sera analyzed by a titration enzyme-linked immunosorbent assay (ELISA) using plates coated with the ECDHER2 or Id. The presence of antibodies was detected using alkaline-phosphatase-labeled anti-(mouse IgG). Values represent the average of duplicate dilutions of serum required to yield an absorbance of 0.1 (410 nm).

<table>
<thead>
<tr>
<th>Cells injected</th>
<th>Antibody tested</th>
<th>Mouse number</th>
<th>Antibody level after challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3 days</td>
<td>6 days</td>
</tr>
<tr>
<td>38C13-HER2/neu</td>
<td>Anti-(human HER2/neu)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Anti-(mouse Id)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Anti-(mouse Id)</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Anti-(mouse Id)</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Anti-(mouse Id)</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>38C13</td>
<td>Anti-(mouse Id)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Anti-(mouse Id)</td>
<td>2</td>
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</tr>
<tr>
<td></td>
<td>Anti-(mouse Id)</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
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<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Anti-(mouse Id)</td>
<td>5</td>
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</tr>
</tbody>
</table>

Tumor response

Table 4 Comparison of anti-(human HER2/neu) and anti-Id levels in mice bearing s.c. 38C13-HER2/neu or 38C13 tumors. Groups of 5 mice injected in the right flank with 10³, 10⁴, 10⁵ or 10⁶ 38C13-HER2/neu or 38C13 cells were bled 12 days after the injection of the cell and the sera analyzed by a titration ELISA using plates coated with the ECDHER2 or Id. The presence of antibodies was detected using alkaline-phosphatase-labeled anti-(mouse IgG). Values represent the average of duplicate dilutions of serum required to yield an absorbance of 0.1 (410 nm). Tumor growth was monitored and measurements were recorded three times per week with a caliper. Growth was classified as progression (P) or regression (R).

<table>
<thead>
<tr>
<th>Cells injected</th>
<th>Antibody tested</th>
<th>Mouse number</th>
<th>Antibody level and tumor response after injection of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>10³ cells</td>
</tr>
<tr>
<td>38C13-HER2/neu</td>
<td>Anti-(human HER2/neu)</td>
<td>1</td>
<td>0 P</td>
</tr>
<tr>
<td></td>
<td>Anti-(mouse Id)</td>
<td>2</td>
<td>0 P</td>
</tr>
<tr>
<td></td>
<td>Anti-(mouse Id)</td>
<td>3</td>
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<td>0 R</td>
</tr>
<tr>
<td></td>
<td>Anti-(mouse Id)</td>
<td>5</td>
<td>0 P</td>
</tr>
<tr>
<td>38C13</td>
<td>Anti-(mouse Id)</td>
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<td>0 P</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>Anti-(mouse Id)</td>
<td>5</td>
<td>0 P</td>
</tr>
</tbody>
</table>

may be associated with an effective antitumor response. For mice injected with 10⁴ 38C13-HER2/neu cells, the only mouse showing tumor progression exhibited the lowest anti-Id titer. In the group of mice injected with 10⁵ 38C13-HER2/neu cells, the only mouse showing tumor regression exhibited the highest anti-Id titer. In the group of mice injected with 10⁶ cells, 1 of the 3 mice showing tumor regression exhibited the highest anti-Id titer while the other 2 mice exhibited titers similar (0 or 50) to those of mice showing tumor progression. No anti-Id was detected in mice injected with 10³ 38C13-HER2/neu even though 2 mice showed tumor regression. However, we should stress that, at day 12, tumor regression had not yet begun in mice injected with 10³ or 10⁴ cells, while mice injected with 10⁵ or 10⁶ cells already showed clear signs of regression or progression. Thus, the day 12 response may not accurately reflect the association between anti-Id titers and the antitumor response.
Table 4 also shows that no anti-Id was seen in sera collected from day-12 mice injected with 10^3 or 10^4 parental 38C13 cells. However, increasing the injection dose of 38C13 cells resulted in detectable titers of anti-Id antibodies in 3/5 (60%) mice challenged with 10^5 38C13 and in 5/5 (100%) of mice challenged with 10^6 38C13. These anti-(mouse Id) titers, however, are clearly lower than that those found in mice challenged with 10^4, 10^5, 10^6 38C13-HER2/neo and were not associated with tumor regression.

We also studied the anti-(human HER2/neo) and anti-Id response elicited by day 12 in mice injected with 10^3, 10^4, 10^5 or 10^6 38C13-HER2/neo i.v. (Table 5). Interestingly, anti-(human HER2/neo) titers are present in 4/5 mice injected with 10^3 38C13-HER2/neo; however, in contrast with what was seen in mice injected s.c., increasing the injection dose did not increase the anti-(human HER2/neo) titers. No anti-Id response was detected in any mice injected with 10^3, 10^4 or 10^5 38C13-HER2/neo cells and only a modest response was observed in 3/5 (60%) of mice injected with 10^6 38C13-HER2/neo cells.

In vivo tumor expression of HER2/neo and Id as detected by flow cytometry

Flow-cytometry analysis of freshly isolated cells from 15-day-old s.c. (Fig. 7A, B) or metastatic (Fig. 7C, D) 38C13-HER2/neo tumors growing in cervical lymph nodes from four different mice inoculated with 10^6 cells showed persistence of cell-surface expression of human HER2/neo in all tumors. However, the level of surface expression of human HER2/neo determined immediately following removal of tumor from the mice appears decreased compared with cells maintained in tissue culture. After 1 week in culture the level of human HER2/neo detected markedly increased (Fig. 7E–H) although, in some cases, not to the same level seen with cells maintained continuously in tissue culture. The level of surface expression of Id from the freshly isolated tumors described above appears to be identical to that of cells maintained in tissue culture (data not shown). Similar results have been observed with cells isolated from eight additional continuously growing s.c. 38C13-HER2/neo tumors dissected from different mice injected s.c. and from eight additional metastatic tumors growing in the lymph nodes of mice injected i.v. (data not shown). We have also confirmed that the level of HER2/neo and Id expression is similar among three different metastatic tumors growing in the same mouse and from four tumors from different mice injected i.v. (data not shown). The above results suggest that in vivo selection of variants lacking the expression of human HER2/neo or Id does not occur.

**Discussion**

The 38C13 B-cell lymphoma was successfully transduced with a retroviral construct containing the full-length cDNA encoding human HER2/neo. We found that the transduced cells show stable high-level surface expression of both TAA: human HER2/neo and Id. We also found no secretion of soluble HER2/neo. ECD^HER2 is known to be released by some cancer cells that overexpress HER2/neo [24, 32, 47] and elevated ECD^HER2 serum levels have been described in patients with breast cancer [21, 32]. The secretion of ECD^HER2 has been reported to be a drawback for anti-HER2/neo therapy in humans. In fact, the dose of the rhu MAb HER2, now in clinical use, provides adequate serum concentrations in all patients except those with serum levels of tumor-shed ECD^HER2 of 500 ng/ml or more [2, 3]. Tumor-shed Id has been described as a significant limitation for antibody-based therapeutic approaches targeting the Id expressed on lymphomas [28]. Mice bearing the 38C13 tumors accumulate only a small amount of antibodies bearing the Id during the first week following injection.

<table>
<thead>
<tr>
<th>Cells injected</th>
<th>Antibody tested</th>
<th>Mouse number</th>
<th>Antibody level after injection of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>10^3 cells</td>
</tr>
<tr>
<td>38C13-HER2/neo</td>
<td>Anti-(human HER2/neo)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>150</td>
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<td></td>
<td></td>
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<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>450</td>
</tr>
<tr>
<td>Anti-(mouse Id)</td>
<td></td>
<td>1</td>
<td>0</td>
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<td></td>
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<td>2</td>
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<td>5</td>
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The presence of antibodies was detected using alkaline-phosphatase-labeled anti-(mouse IgG). Values represent the average of duplicate dilutions of serum required to yield an absorbance of 0.1 (410 nm).
Fig. 7A–H Flow-cytometry analysis of 38C13-HER2/neu cells freshly isolated from tumors of 2 mice injected s.c. with 10^6 cells (A, B) or from cervical metastasis of 2 mice injected i.v. with 10^7 cells (C, D). All tumors were harvested 15 days after tumor cell injection. The cells were stained with anti-dansyl human IgG3 (- -) or anti-HER2/neu human IgG3 (---), followed by biotinylated goat anti-human IgG3 and phycoerythrin-labeled streptavidin. The expression of human HER2/neu was compared with the expression detected in 38C13-HER2/neu cells maintained in culture (--). We also tested the expression of HER2/neu after the cells had been cultured in vitro for 1 week. E–H The cells shown in panels A–D respectively after they had been maintained for 1 week in tissue culture.

of a relatively small number of cells, but after the tumor becomes established the level of Id protein detected in serum increases at a logarithmic rate making the treatment of established tumors very difficult [28]. The lack of tumor-shed ECDHER2 in our HER2/neu-expressing cell lines indicates that secretion of this TAA will not interfere with assessment of antibody or antibody fusion protein treatments targeting HER2/neu.

In previous studies, we found that expression of human HER2/neu on the surface of murine CT26 and MC38 adenocarcinomas and EL4 T-cell lymphoma does not significantly change the in vivo growth properties or morphology of these cells [31]. These in vivo properties dramatically contrast with the cell death and complete tumor regression, resulting in permanent immunity to further challenge observed in s.c. 38C13-HER2/neu tumors. This regression was effected through immunological mechanisms since it was not observed in syngeneic immunodeficient mice. These differences in growth characteristics appear to be a consequence of either the nature of the parental cells and/or the mouse strain rather than the level of HER2/neu expression, which is similar in all tumor models (data not shown). The same HER2/neu expression vector, MoMuLV-based retroviral vector, including the neo gene under the control of the SV40 promoter, was used to produce all the HER2/neu-expressing cell lines.

The ability to transfer immunity into naïve mice using serum from immune mice suggests that an antibody-mediated mechanism is at least partially responsible for tumor rejection and the maintenance of immunity. This is consistent with other reports that vaccinations using either the Id protein, or DNA encoding for Id, induced tumor protection that can be largely attributed to humoral rather than cellular immunity [9, 40]. We also showed that immunity can be transferred by splenocytes from immune mice; however, this is also consistent with the humoral mechanism because 40% of splenocytes are
B cells [19]. However, as 35% of splenocytes are T cells [19] we can not exclude the possibility that a T cell immune response might also provide antitumor immunity. We have found that transduction of this tumor and other murine tumor models with human HER2/neu does not decrease the level of expression of MHC class I (M.L. Penichet et al., unpublished results), suggesting that those cells may be able to elicit targeted cytotoxic T cell response. Further studies are required to define the role (if any) of cellular immunity in tumor rejection and immunity.

Having shown that immunity can be transferred by sera, we characterized the anti-(human HER2/neu) and anti-Id responses. We detected an anti-human HER2/neu antibody response in mice bearing HER2/neu-expressing tumors (s.c. and i.v.). A similar humoral response has also been described in mice bearing primary s.c. or metastatic CT26-HER2/neu, MC38-HER2/neu, and EL4-HER2/neu tumors, although in these models tumor regression was not observed [31]. Although the anti-(human HER2/neu) antibodies elicited against the s.c. 38C13-HER2/neu tumors may play a role in tumor rejection, we have found no correlation between the level of anti-HER2/neu titers and the fate of the tumor (regression or progression). We also found that continuously growing tumors maintain human HER2/neu expression, suggesting that variants lacking the expression of human HER2/neu are not selected. An alternative possibility is that the presence of foreign antigens such as HER2/neu on the surface of 38C13 serves as an adjuvant to facilitate a humoral immune response against other antigens such as the Id. Anti-Id therapy has been successful in the 38C13 model [6, 7, 25, 30]. In fact we have found higher titers of anti-Id antibodies in mice bearing s.c. 38C13-HER2/neu than in mice bearing s.c. 38C13 tumors. Furthermore higher anti-Id titers appear to correlate with tumor regression. However anti-Id antibodies were not detected in some of the mice showing regressing s.c. 38C13-HER2/neu tumors. It is possible that, in these mice, Id shedding by the 38C13 tumors [28] leads to immune complexes decreasing the concentration of anti-Id antibodies in blood and resulting in underestimation of the anti-Id response.

Although the ability to elicit an anti-Id immune response might explain the spontaneous tumor regression, we can not exclude the possibility that other antibodies with unknown specificity have been elicited and that these are partially or totally responsible for tumor regression and immunity. In favor of this hypothesis is the observation that the rejection of 38C13-HER2/neu results in immunity to further challenge not only with the parental cell line 38C13, but also with V1, a cell line that is negative for Id expression [38] and insensitive to treatment with anti-Id antibodies [40]. This observation suggests that antitumor immunity generated after regression of 38C13-HER2/neu is directed against one or more common antigens shared by 38C13-HER2/neu, 38C13 and V1. Such antigens may be known receptor molecules such as CD19 and CD40, which have been successfully used as targets of antibody-based therapy in B cell lymphoma [43] or may be unknown antigens. If unknown antigens are the targets, hybridomas from splenocytes of immune mice may provide novel and effective antibodies for the therapy of B cell lymphoma. Several mechanisms have been described to explain the antitumor activity of anti-(B cell lymphoma) antibodies such as anti-Id, anti-CD19 or anti-CD40, including antibody-dependent cell-mediated cytotoxicity (ADCC) [6, 7, 25] as well as antibody-mediated inhibition of tumor growth [43]. Further studies are required to define the mechanism of action of the antitumor activity present in the sera of mice that have rejected the 38C13-HER2/neu tumor.

Xenogenization is a term used to describe attempts to make tumor cells antigenically foreign to their host [22, 23] and includes the expression of foreign antigens such as viral antigens on the surface of tumor cells to potentiate the host immune reaction against the tumor. The rat fibrosarcoma KMT-17 infected with nonlytic murine leukemia virus (Friend virus), like 38C13-HER2/neu, regresses spontaneously in the syngeneic host after an initial period of growth and induces protective immunity to noninfected homologous tumor cells [33]. The mechanism of rejection is not fully understood; however, it was found that surface expression of CE7, a non-viral TAA, is strongly enhanced following viral infection. This enhanced expression may stimulate a strong antitumor response, resulting in acquisition of resistance to parental KMT-17 [33]. Although we did not find that alteration in the expression of the Id in the transductant, we cannot rule out the possibility that there is enhanced expression of other unknown 38C13 TAA antigens.

In addition to expressing HER2/neu, 38C13-HER2/neu cells also express the product of the neo gene, the neomycin phosphotransferase, and this phosphotransferase activity can induce changes in the cells [44] which might result in higher immunogenicity. In fact, the transduction of 9L rat glioma with a neo gene was associated with a decreased in vivo tumor growth in immunocompetent animals, although the mechanism responsible was not defined [41]. Although neomycin phosphotransferase is expressed in the intracellular compartment, it is a bacterial gene product and thus potentially can serve as an immunogen. Even though the neo gene has been used in vast numbers of in vivo experiments without eliciting an immune reaction, 1 patient receiving multiple infusions of gene-modified T lymphocytes was shown to develop anti-neo and anti-(herpes thymidine kinase) cell-mediated immune responses that coincided with rapid disappearance of transduced cells in vivo [8]. Human HER2/neu, the other xenograft antigen that we have expressed, shows more than 90% homology with the rodent HER2/neu [46]. Despite this high degree of homology, its expression appears to be sufficient to elicit an anti-(human Ig) humoral immune response and may trigger the antitumor activity. Thus, the antitumor immune reaction may be elicited by increased expression of TAA and/or by the expression of the xenoantigens human HER2/neu and/or the neo gene.
product, indeed, it is possible that the xenoantigens act as
adjuvants to potentiate the immune response to other
TAAs.

Our data suggest that the parental 38C13 cells can be
immunogenic and that a s.c. inoculation of a very high
dose of 38C13, such as 10^9 cells, is able to trigger a
protective immune response in a subset (10%-20%) of
mice. This contrasts with previous observations that s.c.
inoculation of increasing numbers of 38C13 tumor cells
proportionally shortens the mean survival of injected
animals. However, we should stress that these observa-
tions were made using doses from 10^5 to 10^6 38C13-
tumor cells [6, 7, 25] and we have also found these lower
doses to yield tumors in all animals. Apparently a
threshold level of antigen must be exceeded in order to
elicit a protective immune response. This spontaneous
immune reaction against 38C13 appears to be enhanced
in the transductants. The xenoantigen(s) role as an
adjunct is supported by the finding of significantly higher
titerers of anti-Id antibodies in mice bearing s.c. 38C13-
HER2/neu than in mice bearing s.c. 38C13 tumors.

We speculate that regression of 38C13-HER2/neu tu-
mor was observed more frequently with larger tumor cell
challenges because increased antigenic stimulation results
from larger initial antigenic doses. This strong response
may be able to eliminate the tumor before dissemination
into the lymph nodes. In contrast, lower doses of cells fail
to elicit efficient early stimulation of the immune response,
giving the tumors the opportunity to disseminate and
colonize most of the lymph nodes leading to their dys-
function. The secondary lymphoid organs are critical for
both B- and T-cell-mediated immunity [13] and their
dysfunction would decrease the antitumor reaction, al-
lowing the tumor to progress. However, it should also be
noted that effective antitumor responses are also not elicited by tumors that do not colonize the lymph nodes. It
has been suggested that growing tumors are able to sup-
press the immune response against them [34]. It is possible
that, in the absence of initial strong stimulation, the
growing tumor is able to decrease the subsequent immune
response to its presence.

Our results suggest that, under certain conditions,
expressing antigens of different species (xenoantigens)
on B cell lymphomas may be a useful therapeutic ap-
proach to eliciting an effective antitumor immune
response. This can be achieved by vaccination with tumor
cells expressing the xenoantigen, an approach that has
shown benefits in ovarian cancer patients [27], or by
in vivo transduction of B cell lymphomas with genes
encoding xenoantigens. The presence of the foreign
antigen on the surface of the B cell lymphomas may induce
strong stimulation of the patient's formerly quiescent
immune system. Once stimulated, the patient's immune
repertoire may be directed against other TAA, resulting
in the immune-mediated clearance of all B cell lymphoma
cells.

The results obtained with s.c. implantation of cells
contrast markedly with those obtained after i.v. injection
of 38C13-HER2/neu. Following i.v. injection, all mice
developed a disseminated malignant disease leading
to death. Although the tumors elicited following i.v.
 injection maintain HER2/neu expression, they failed to
elicit the strong anti-(human HER2/neu) and anti-Id
humoral immune response observed in mice bearing s.c.
38C13 HER2/neu. This observation may not be sur-
prising because it is well known that the s.c. route is
superior to the i.v. route in eliciting an immune response
in mice [15]. In addition, the i.v. injection of cells results
in metastases to the lymph nodes throughout the body,
which may seriously compromise the function of these
important secondary immune organs [13] resulting in
poor antitumor immunity as discussed above.

38C13-HER2/neu may indeed be a useful model for
evaluating the immunological efficacy of antibody or
antibodies fusion proteins. We have shown that an
effective immune response eliminates the tumor. This
effective response is usually not elicited in mice injected
s.c. with low doses (i.e. 10^5) of tumor cells or in mice
injected i.v. with any dose. Tumors elicited following
both s.c. and i.v. injection maintain a high level of ex-
pression of human HER2/neu. In addition, the presence
of anti-HER2/neu antibodies does not preclude the use
of these mice for therapies targeting HER2/neu since the
treatment can be started shortly after the injection of
tumor cells when there is little or no humoral response.
Indeed it may be possible to target the tumor in the
presence of an anti-HER2/neu response since (hu-
man carinoembryonic antigen, CE) was able to target
MC38 transduced with human CE (MC38-cen2), which elicits a murine anti-(human CE) response [14].
We also found that both anti-HER2/neu scFv and
anti-HER2/neu IgG3 target CT26-HER2/neu growing in
immunocompetent mice, a cell line that also elicits a
murine anti-(human HER2/neu) immune response
(M. L. Penichet et al., unpublished results). We thus
have the opportunity to use both HER2/neu and the Id
as targets for our antibody fusion proteins. The chal-
lenge now is to develop a strategy that will make it
possible to elicit an effective immune response to tumor
after i.v. injection and/or to low doses of tumor cells
injected s.c.

Acknowledgements 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-CA009120-
25 from NC1 (NIH) and Cancer Center Core grant
CA-16042 (UCLA). We are grateful to Drs. Ronald and Shoshana Levy for
cell lines 38C13, the V1 variant of 38C13 and the hybridoma A1-2,
to Dr. James D. Marks for ECDHER2
and to Dr. Donald Morrison
for assistance with statistical analysis. We also acknowledge Dr.
Koteswara Chintalacharuvu, Dr. Manou Seyedeh, and Dr. David
Beenhoeur for careful review of the manuscript and useful
suggestions.

References


A Single-Chain IL-12 IgG3 Antibody Fusion Protein Retains Antibody Specificity and IL-12 Bioactivity and Demonstrates Antitumor Activity

Lisan S. Peng, Manuel L. Penichet, and Sherie L. Morrison

IL-12 is a heterodimeric cytokine with many actions on innate and cellular immunity that may have antitumor and antimetastatic effects. However, systemic administration of IL-12 can be toxic. Tumor-specific Abs provide a means to selectively target a metastatic/residual nodule and deliver therapeutic quantities of an immunostimulatory molecule like IL-12 with lower systemic levels and ideally, toxicity. We report the construction and characterization of an Ab fusion protein in which single-chain murine IL-12 is fused to an anti-Her2/neu Ab at the amino terminus (mscIL-12.her2.IgG3). The use of single-chain IL-12 in the fusion protein simplifies vector construction, ensures equimolar concentrations of the two IL-12 subunits, and may confer greater stability to the fusion protein. SDS-PAGE analysis shows this 320-kDa protein is secreted and correctly assembled. FACS analysis demonstrates that this fusion protein binds to cells transfected with the Her2/neu Ag, thus retaining Ab specificity; this fusion protein also binds to a cell line and to PHA-activated PBMC that express the IL-12R, thus demonstrating cytokine receptor specificity. T cell proliferation assays and NK cytotoxicity assays demonstrate that this fusion protein exhibits IL-12 bioactivity comparable to recombinant murine IL-12. In vivo studies demonstrate that this fusion protein has antitumor activity. These results are significant and suggest that this IL-12 Ab fusion protein can effectively combine the therapeutic potential of IL-12 with the tumor-targeting ability of the Ab and may provide a viable alternative to systemic administration of IL-12. The Journal of Immunology, 1999, 163: 250–258.

The management of residual and metastatic disease is a central problem in the treatment of cancer. Chemotherapeutic strategies can be effective, but are frequently limited by various toxicities. Therefore, additional modalities are needed to achieve disease containment or elimination. One approach has been to attempt to elicit a specific immune response by the host against tumor-associated Ags. Treatment with cytokines has been shown to render some nonimmunogenic tumors immunogenic, activating a protective immune response (1–4). However, when cytokines are given systemically there are frequently problems with toxicity that make it impossible to achieve an effective dose at the site of the tumor (5, 6). Ideally, strategies which increase the cytokine concentration at the site of the tumor and allow for lower systemic levels should be more effective.

Two approaches to achieving high levels of cytokine at the site of the tumor have been direct injection of cytokine into the tumor or transfer of the gene encoding the cytokine into tumor cells (7). While both methods have been shown to be effective, they also have significant limitations: direct injection into micrometastases is not possible, and currently gene transfer involves ex vivo manipulation of tumor cells, which makes treatment of large numbers of patients difficult and costly. Abs provide an alternative specific delivery vehicle in which tumor-specific Abs can be used to selectively target a metastatic/residual nodule and deliver an immunostimulatory molecule like a cytokine. The specific targeting should make it possible to elicit a systemic tumor-specific immune response without accompanying systemic toxicity.

There are many different types of tumor-associated Ags: oncofetal Ags (e.g., carcinoembryonic Ag (8)), Ags expressed on cells at a particular stage of differentiation (e.g., IL-2R (9)), growth factor receptors (e.g., transferrin receptor (10)), oncogene products (e.g., c-myc (11)), and the Id expressed by the surface Ig of lymphoma cells (12). These tumor-associated Ags distinguish normal from tumor tissue and have been used as targets for cancer therapy (13–17). Her2/neu, also known as c-erbB-2, is a cell surface onco gene product that is amplified and/or overexpressed in 25–30% of human breast and ovarian cancers with this overexpression associated with poor prognosis (18, 19). Humanized anti-Her2/neu has been shown to be an effective therapeutic agent in clinical trials (20). These trials demonstrate that metastatic breast cancer can be effectively targeted through the Her2/neu Ag and suggest that Abs specific for Her2/neu would be effective vehicles for targeting cytokines to the sites of the tumors.

Several different cytokines are attractive candidates for enhancing tumor-specific immune responses. IL-2 induces the proliferation of T cells, supports the growth of Ag-specific T cell clones, and enhances the activity of T and NK cells (21). Fusion of IL-2 to Abs specific for tumor-associated Ags such as ganglioside GD2 and the Id of a murine lymphoma has resulted in fusion proteins that have shown much promise as agents for stimulating tumor-specific immune responses (22–27). Indeed, an IL-2-Ab fusion protein specific for GD2 was able to generate an immune response.
that eliminated metastatic disease in a murine model of melanoma (22).

Another cytokine that has great potential for use in tumor immunotherapy is IL-12. IL-12 is a heterodimeric cytokine with many actions on innate and cellular immunity that may have antitumor and antitumorastatic effects. IL-12 can activate T and NK cells, induce the production of IFN-γ, and stimulate naive CD4+ T cells to differentiate toward the Th1 phenotype (28, 29). A Th1 response involves the secretion of a cytokine profile that activates cytotoxic T cells and macrophages, which could be desirable in an antitumor immune response. In addition, IL-12 may act through nitric oxide to cause cell-cycle arrest of tumor cells (4), and through induction of inducible protein-10 to inhibit angiogenesis (30).

Bioactive IL-12 requires the expression of two separate genes, p40 and p35, and correct heterodimer assembly (31). To address this issue, Gillies et al. have recently reported the construction of an Ab-IL-12 fusion protein in which the p35 subunit was fused to the carboxyl terminus of an Ab; the p40 subunit was expressed as a separate polypeptide that must then assemble with the p35 subunit. Although this IL-12/Ab fusion protein was functional, the IL-12 bioactivity was 2-fold lower than rIL-12 (32). An alternative that eliminates the need to assemble two independently produced peptides is to express IL-12 as a single chain with the p40 and p35 subunits joined by a flexible linker. We have now used this alternative approach and constructed an Ab fusion protein in which murine single-chain (msc) IL-12 (p40 linker:p35) is fused to an anti-Her2/neu Ab at the amino terminus of the H chain (mscIL-12/Her2.IgG3). Importantly, this fusion protein retains Ab specificity, exhibits IL-12 bioactivity comparable to recombinant murine (m) IL-12, and demonstrates antitumor activity in vivo.

**Materials and Methods**

**Cell lines and reagents**

P3X63Ag8.653 cells (American Type Culture Collection, Manassas, VA), CT26 cells (murine colon adenocarcinoma cells kindly provided by Young Chul Sung, Pohang University, Korea), and CT26/Her2 cells (developed in our laboratory by transfection of CT26 cells with the cDNA encoding Her2/neu using methods previously described (33)) were cultured in IMDM supplemented with 5% bovine calf serum, l-glutamine, penicillin, and streptomycin. K562 cells (American Type Culture Collection) were cultured in RPMI 1640 supplemented with 10% FBS, sodium pyruvate, HEPES, and d-glucose. Kit225/Kβ cells (kindly provided by Jim Johnston, DNAX, Palo Alto, CA) were maintained in RPMI 1640 supplemented with 10% FBS and 100 IU recombinant human (h) IL-2 (mll) (kindly provided by Chiron, Emeryville, CA). rmIL-12 reference standard was kindly provided by Stanley Wolf (Genetics Institute, Cambridge, MA).

**Mice**

Female 6- to 8-week-old BALB/c mice were obtained from Taconic Farms (Germantown, NY) and conventionally housed. All experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Ab expression vectors**

**her2.IgG3.** The variable L and H chain domains were obtained from the plasmid pAK19 containing the humanized humAbD5-8 Ab (generously provided by Paul Carer, Genentech, South San Francisco, CA) (34, 35) and cloned as previously described (36) into mammalian expression vectors for human κ L chain and IgG3 H chain, respectively.

**mscIL-12/her2.IgG3.** The cDNA for mscIL-12 (p40 subunit linked by a (Gly,Ser)5 flexible linker to the p35 subunit from which the first 22 aa (leader sequence) were deleted) was generously provided by Richard Mul-ligan (Harvard Medical School, Boston, MA) as plasmid pSP72-4mlL 2-p40 linker;Δp35. mscIL-12 was amplified from the plasmid by PCR using the sense primer 5'-CCCCAAGCTTGTATACCCATGCCT CTCAGAACCTAACC-3' and the antisense primer 5'-CCCGAATTCTG TAACCCGGGAGCTCAGATAGCC-3'. The PCR product was cloned as a HindIII/Hpal fragment to the 5' end of a cassette encoding the (Gly,Ser)5 linker sequence of Huston et al. (37) fused to the anti-Her2/neu V_{H} sequence. The resulting mscIL-12 linker V_{H} coding sequences were excised as an EcoRV/Nhel fragment and cloned into an expression vector for human IgG3 H chain (38).

**Recombinant Ab expression, immune precipitation, and purification**

Transfection, expression, and purification of the recombinant Abs were performed as previously described (39) to obtain mscIL-12/her2.IgG3. Briefly, 1 × 10^5 P3X63Ag8.653 syngeneic mouse cells were transfected via electroporation with 10 μg of each of the mscIL-12/her2.IgG3 H and anti-Her2κ L chain expression vectors (linearized with PvuII). Transfected cells were plated at 2 × 10^6 cells/well in a flat-bottom 96-well tissue culture plate and selected with the addition of 10 mM bistidinol (Sigma, St. Louis, MO) on days 3 and 5 after transfection. Wells were screened for Ab secretion after 10–14 days by ELISA using 96-well flat-bottom plates coated with goat anti-human IgG (Zymed, South San Francisco, CA). Supernatant from the transfected cells was applied, followed by the addition of goat anti-human κ conjugated with alkaline phosphatase (Sigma). Binding was detected by the addition of phosphatase substrate (para-nitrophenyl phosphate, disodium: Sigma), and positive wells were expanded.

To determine the size and assembly pattern of the secreted recombinant mscIL-12/her2.IgG3, supernatants from cells grown overnight in medium containing [35S]methionine (Amersham, Piscataway, NJ) were immunoprecipitated with polyclonal rabbit anti-human IgG and rabbit anti-human κ (produced by Letitia A. Wims in our laboratory), followed by staphylococcal protein A (IgG Sorb; The Enzyme Center, Malden, MA). Precipitated Abs were analyzed on SDS-polyacrylamide gels in the presence or absence of reducing agent (2-ME).

For the purification of mscIL-12/her2.IgG3, high producing clones were expanded in roller bottles in IMDM plus 1% fetal calf serum plus Glutamax (Life Technologies, Rockville, MD), and cell-free culture supernatant was collected. Culture supernatants were passed through a protein A column, the column was washed with 10 ml PBS, and the proteins were successively eluted with 2 ml of 1 M citric acid, pH 4.5, 5 ml of 0.1 M glycine, pH 2.5, and 2 ml of 0.1 M glycine, pH 2.0. The eluted fractions were neutralized immediately with 2 M Tris-HCl, pH 8.0. The fractions were concentrated using Ultra-free-15 filters (Millipore, Bedford, MA) with a cut-off of 100 kDa and diaлизed. Using this method, 2 L of culture supernatant yielded ~0.8 mg mscIL-12/her2.IgG3.

**Assays of binding to Her2/neu Ag and IL-12R**

**Ag binding.** CT26 or CT26/Her2 were incubated with mscIL-12/her2.IgG3, her2.IgG3, or dapsyl.IgG3 (an IgG3 isotype control Ab specific for the hapten dansyl) for 1 h at 4°C. The cells were washed and incubated 2 h at 4°C with PE-labeled goat anti-human IgG (PharMingen, San Diego, CA) and analyzed by flow cytometry. Analysis was performed with a FACSScan (Becton Dickin-son, Mountain View, CA) equipped with a blue laser excitation of 15 mW at 488 nm.

**Persistence of Ab binding at the cell surface.** CT26/Her2 cells were incubated with mscIL-12/her2.IgG3, her2.IgG3, or dansy1.IgG3. The cells were washed and incubated at 37°C in culture medium. At different time points (0, 1, 2, 4, and 24 h), an aliquot of cells was removed and stained by flow with PE-conjugated anti-human IgG for FACS analysis. The mean fluorescence was calculated as a percentage of the maximum mean fluorescence at time zero.

**Binding to IL-12R.** Kit225/Kβ cells, a subclone of the human T leukemia cell line that expresses the IL-12R (40), were incubated with her2.IgG3 or mscIL-12/her2.IgG3. Binding was assayed by staining with PE-conjugated anti-human IgG followed by FACS analysis. In a second assay, PHA-activated PBMC were incubated with her2.IgG3 or mscIL-12/her2.IgG3. PBMC have been shown to express IL-12Rs following activation with PHA and IL-2 (41). Binding was assayed by staining with PE-conjugated anti-human IgG followed by FACS analysis.

**Proliferation assays**

Proliferation assays were performed as previously described (42). PBMC were isolated from normal blood donors by Ficoll-Hypaque density cen-trifugation (Ficoll-Paque, Pharmacia, Piscataway, NJ). These cells were then depleted of monocytes by plastic adherence, and nonadherent cells
were resuspended at $5 \times 10^6$ cells/ml in supplemented medium [1:1 complete RPMI 1640:complete DMEM plus 5% human AB serum (Irvine Scientific, Santa Ana, CA); 10 mM HEPES, 0.006% (w/v) L-arginine monohydrate, and 0.1% (w/v) dextrose] containing 2 µg/ml PHA-P (Difco Laboratories, Detroit, MI) and were cultured for 3 days. Cells were then split 1:1 with fresh supplemented medium containing 20 IU/ml rhIL-2 (kindly provided by Chiron Corporation) and incubated for a further 24–48 h. The PHA blasts were then washed with acidified RPMI 1640, pH 6.4, and rested in RPMI 1640 plus 0.5% human AB serum for 3–4 h. The cell concentration was adjusted to 2–10^6 cells/ml in supplemented media. Neutralizing anti-IL-2 Ab (BioSource International, Camarillo, CA) was added at 1 µg/ml to block IL-2-induced proliferation.

Serial 1:3 dilutions of equivalent protein concentrations of mIL-12, mscIL-12.her2-lgG3, and her2-lgG3 were made in supplemented medium over a range of 36 ng/ml to 16 pg/ml. Next, 50 µl cell suspension was mixed with 50 µl mIL-12, mscIL-12.her2-lgG3, or supplemented medium in triplicate in a flat-bottom 96-well tissue culture plate. After 48 h of culture at 37°C, 5% CO_2, proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)/phenazine methosulfate (PMS) assay (Promega, Madison, WI), and plates were read at OD_990.

Enhanced NK activity of PBMC

These assays were performed according to the methods of Hatam et al. (43) with modifications. Briefly, effector PBMC were isolated as described above, then resuspended in RPMI 1640 plus 10% FBS at 1–2×10^6 cells/ml. mIL-12 at 3 ng/ml, an equivalent IL-12 concentration of mscIL-12.her2-lgG3, or an equivalent Ab concentration of her2-lgG3 were added to the PBMC and incubated for 16–18 h at 37°C, 5% CO_2. The cell concentration was then adjusted to 0.5–1×10^6 cells/ml. Target K562 cells (2×10^5) were washed two times with serum-free RPMI 1640, then resuspended in 1 ml Diluent C (Sigma). Then, 4 µM PKH67 was prepared by diluting the stock solution (Sigma) in Diluent C. The cell suspension and dye were mixed in equal volumes (1 ml each) in a polystyrene tube and incubated at room temperature for 2 min. An equal volume (2 ml) of FBS was added to stop the labeling reaction. The cells were washed three times with RPMI 1640 plus 10% FBS and resuspended at 1–2×10^5 cells/ml in RPMI 1640 plus 10% FBS.

For the NK cytotoxicity assay, 100 µl effector PBMC and 100 µl PKH67-labeled K562 cells were added to polystyrene 12 × 75 mm tubes to create E:T ratios of 50:1 and 100:1 and were incubated for 4 h at 37°C, 5% CO_2. At the end of the incubation, 0.5 ml isotonic propidium iodide (PI) at 5 µg/ml (Sigma) was added to each tube and immediately analyzed by FACS. Spontaneous cell death was determined by incubating either targets or effectors alone.

FACS analysis was performed with a FACScan (Becton Dickinson) equipped with a blue laser excitation of 15 mW at 488 nm. The two fluorochromes, PKH67 and PI, were electronically compensated using PKH67-labeled targets alone and unstained target cells whose membrane had been permeabilized by treatment with 0.1% Tween-20 in PBS for 10 min at 37°C. These cells were then washed twice and 0.5 ml of isotonic PI added before FACS analysis. Data were collected in list mode and analyzed using Cell Quest software (Becton Dickinson). At least 2000 target events were collected per sample. Percent cytotoxicity was calculated as (number of dead targets)/(total number of targets) × 100.

In vivo antitumor activity

A total of 1×10^6 CT26/her2 cells in 0.15 ml PBS were injected s.c. into the right flank of syngeneic BALB/c mice on day 0. One group of mice was treated i.v. with mscIL-12.her2-lgG3 (at a concentration equivalent to 1 µg IL-12/day), her2-lgG3 (at a concentration equivalent to the Ab concentration of mscIL-12.her2-lgG3 administered/day), or PBS for 5 days beginning on day 1. A second group of mice was similarly treated beginning on day 6. In each group, 10 mice per treatment arm were used. Tumor growth was monitored and measured with a caliper every other day beginning on day 6 and continuing until day 20. At that point, all mice were euthanized and the tumors were harvested and weighed.

Results

**Design and expression of single-chain (sc) IL-12 Ab fusion protein**

The construction of her2-lgG3 and vectors for the production of H chain fusion proteins was previously described (36). For the present studies, we elected to use mIL-12 in our fusion protein because mIL-12 is biologically active on activated murine and human T and NK cells, while murine T and NK cells do not respond to hIL-12 (44). mscIL-12 was amplified from plasmid pSP72.mIL-12.p40 linker Δp35 and cloned at the amino terminus of the V_4 region of her2-lgG3 (Fig. 1). A flexible (Gly_Ser)_3 linker was positioned between IL-12 and the V region to facilitate both correct folding of the Ab and IL-12 and simultaneous Ag and IL-12R binding. The mscIL-12.her2-lgG3 H chain and κ L chain were cotransfected into P3X63Ag8.653 myeloma cells, and stable transfectants secreting Ab were selected using the anti-human IgG ELISA described in Materials and Methods.

To determine the molecular mass and assembly of the secreted Ab, the cells were grown overnight in [35S]methionine. Anti-human IgG and anti-human κ followed by insoluble protein A were added to the supernatant and cell lysates, and the immunoprecipitates were analyzed by SDS-PAGE. In the absence of reducing agents, IgG3 migrates with an apparent molecular mass of 170 kDa, while mscIL-12.her2-lgG3 is about 230 kDa, the expected molecular mass of the fusion protein (Fig. 2A). Following treatment with the reducing agent 2-ME, L chains migrating with an apparent molecular mass of ~25 kDa are seen for both proteins. However, the IgG3 has a H chain of ~60 kDa, while mscIL-12.her2-lgG3 has a H chain of ~135 kDa. Thus, proteins of the expected molecular mass are produced, and fusion of scIL-12 to
her2.IgG3 does not appear to alter the assembly and secretion of the H₂L₂ form of the Ab.

**Ag binding and persistence of Ab binding at the cell surface**

The ability of mscIL-12.her2.IgG3 to bind to the Her2/neu antigenic target was examined using flow cytometry. Both mscIL-12.her2.IgG3 (Fig. 3B) and her2.IgG3 (Fig. 3C) specifically bound to CT26/Her2; neither Ab bound to parental CT26 cells (data not shown). Importantly, the same fluorescence intensity was seen with both her2.IgG3 and mscIL-12.her2.IgG3, suggesting that both have similar affinity for Her2/neu. A control IgG3 Ab specific for the hapten dansyl did not bind to CT26/Her2 (Fig. 3A). These data indicate that the fusion of a 75-kDa scIL-12 to the amino terminus of each H chain of her2.IgG3 does not interfere with the ability of the Ab to recognize the Her2/neu Ag.

There is no significant difference in the persistence of Ab bound to the cell surface between mscIL-12.her2.IgG3 and her2.IgG3, with both still showing similar fluorescence intensity at all time points and >30% staining at 24 h (Fig. 4). These results indicate that fusion of the Ab with IL-12 does not affect the dissociation rate, internalization, or degradation of mscIL-12.her2.IgG3 at the surface of the cell compared with her2.IgG3. This suggests that the IL-12 in our fusion protein will be present at the cell surface to activate T and NK cells.

**Binding to the IL-12R**

The ability of mscIL-12.her2.IgG3 to bind to the IL-12R was determined by flow cytometry of both transformed and normal human cells. The mscIL-12.her2.IgG3 bound to KIt25/K6, a subclone of the human T leukemic cell line that expresses the IL-12R (40), while her2.IgG3 did not (Fig. 5A). Neither her2.IgG3 or
FIGURE 5. Binding to IL-12R. Kit225/K6 cells (A), which express IL-12Rα1, resting PBMC (B), or PHA-activated PBMC (C) were incubated with her2.IgG3 (solid line) or mscIL-12:her2.IgG3 (dotted line). Binding was assayed by staining with PE-conjugated anti-human IgG followed by FACS analysis. Note that a shift in a peak means that the entire population of cells now reacts with the IL-12-bearing Ab.

mscIL-12:her2.IgG3 bound to the resting PBMC (Fig. 5B). The mscIL-12:her2.IgG3 bound to the PHA-activated PBMC while her2.IgG3 did not (Fig. 5C). These results show that the IL-12 in the fusion protein is able to bind to the IL-12R.

Proliferation assays

After establishing that mscIL-12:her2.IgG3 was correctly assembled, secreted, and retained the ability to bind both the Her2/neu Ag and the IL-12R, we investigated its biologic activity. All assays of IL-12 biological activity were expressed relative to the IL-12 concentration used (i.e., ng/ml). To obtain the IL-12 concentration of the fusion protein, the fraction of the IL-12-Ab fusion protein that was IL-12 (150 kDa/320 kDa) was multiplied times the protein concentration of the fusion protein. In this way, the biological activity of rIL-12 and IL-12 in the fusion protein could be compared on a per molecule basis. Similarly, to ensure that equivalent Ab concentrations of her2.IgG3 and mscIL-12:her2.IgG3 were used, the fraction of the Ab-fusion protein that was Ab (170 kDa/320 kDa) was multiplied times the protein concentration of the fusion protein to obtain the Ab concentration of the fusion protein. The same concentration of her2.IgG3 was then used as a control.

One of the pleiotropic actions of IL-12 is the ability to induce the proliferation of PHA-activated lymphoblasts. We prepared PHA-activated PBMC and incubated them for 48 h with mIL-12, mscIL-12:her2.IgG3, or her2.IgG3. Proliferation was measured by addition of MTS/PMS. Fig. 6 shows the results from a typical assay. mIL-12 and mscIL-12:her2.IgG3 showed an equivalent mitogenic effect on PHA blasts in a dose-dependent manner. The results are expressed as the mean ± SD of triplicate samples with the background proliferation in medium subtracted. In contrast, her2.IgG3-treated PHA blasts did not show any proliferation. These results indicate that the mitogenic effect of mscIL-12:her2.IgG3 is due to the IL-12 and not to some other effect by the Ab component of the fusion protein.

Enhanced NK activity

IL-12 has been shown to enhance the cytotoxic action of NK cells. We prepared PBMC (shown by FACS to be 8–9% CD56−, data not shown) and incubated them for 16–18 h with 5 ng/ml mIL-12 or an equivalent IL-12 concentration of mscIL-12:her2.IgG3. The PBMC were also incubated with her2.IgG3 at the same Ab concentration as mscIL-12:her2.IgG3 or medium. These effector cells were added to PKH67-labeled K562 target cells at E:T ratios of 100:1 and 50:1, then incubated for 4 h. After this incubation, PI, which intercalates into the DNA of dead cells, was added and FACS analysis performed. Fig. 7A shows a representative FACS result (E:T of 100:1, treated with 5 ng/ml mIL-12) with defined populations of live effectors (lower left), dead effectors (upper left), live targets (lower right), and dead targets (upper right) separated into the four quadrants. The x-axis measures PKH67 fluorescence intensity and the y-axis measures PI fluorescence intensity. A gate was drawn around target cells (PKH67 positive). Figs. 7, B–E show histograms of PI fluorescence intensity among target cells gated as in Fig. 7A. More positively staining cells (right) are dead target cells; less positive cells (left) are live target cells. The percent cytotoxicity was calculated from the histograms. The background cytotoxicity in medium was subtracted to give the percent enhanced cytotoxicity. Fig. 7E shows that mIL-12 and mscIL-12:her2.IgG3 at equivalent IL-12 concentrations of 5 ng/ml and E:T of both 50:1 and 100:1 compared enhanced NK cytotoxicity by ~20%, while her2.IgG3 showed no enhancement. These results indicate that the enhanced cytotoxicity by mscIL-12:her2.IgG3 is due to the IL-12 component of the fusion protein and not to some other effect by the Ab component of the fusion protein and that the IL-12 in the fusion protein has activity comparable to rIL-12.

In vivo antitumor activity

After demonstrating that mscIL-12:her2.IgG3 had in vitro biologic activity comparable to rIL-12, the in vivo antitumor activity was investigated using a CT26/Her2 animal model developed in our laboratory (33).

On day 0, CT26/Her2 cells were injected s.c. into the right flank of BALB/c mice. One group of mice was treated with mscIL-12:her2.IgG3, her2.IgG3, or PBS injected i.v. for 5 days beginning
on day 1, while a second group was similarly treated beginning on day 6 when the tumors averaged 8–9 mm in diameter. Thus, the studies were designed to examine the effect of mscIL-12.her2.IgG3 on both tumor growth and tumor regression. Treatment with mscIL-12.her2.IgG3 slowed the growth of tumors when it began on day 1 (Fig. 8A) and arrested tumor growth when it began on day 6 (Fig. 8C) compared with mice treated with PBS or her2.IgG3. The tumor weights were used as a more objective indicator of tumor size and confirm the results of the caliper measurements (Figs. 8, B and D).

These results demonstrate that mscIL-12.her2.IgG3 has significant antitumor activity in immunocompetent mice. Further studies are in progress to determine whether this effect can be seen in other tumor models and to determine the mechanism of the observed antitumor activity.

Discussion

In these studies, we describe the construction and expression of a novel bioactive mscIL-12 IgG3 Ab fusion protein. In the design of our Ab-IL-12 fusion protein, a number of factors were considered. Although our long-term goal is the production of Ab fusion proteins for therapeutic use in humans, mIL-12 was used for these initial studies because it has activity on both human and murine cells, while hIL-12 has activity only on human cells. The use of mIL-12 makes it possible not only to carry out assays using human PBMC to test biologic activity, but also to perform in vivo studies using immunocompetent mice to examine the effects against Her2/neu-expressing murine tumors.

Previous studies suggested that an accessible N terminus of the p40 subunit is important for IL-12 bioactivity. When Lieschke et al. constructed a scIL-12, the order of the subunits was found to affect the IL-12 biologic activity (45). When the p35 subunit came before the p40 subunit, there was greatly decreased IL-12 activity; in contrast, when the subunits were reversed, with p40 in front of p35, the scIL-12 had biologic activity comparable to hIL-12 (45–47). Similarly, in an OVA-IL-12 fusion protein in which the p40 subunit was fused to OVA, a 50-fold lower IL-12 activity was observed (48). Constraint of the p40 subunit in a fusion protein may disrupt the interaction between IL-12 and the IL-12R. The IL-12R complex consists of two chains, β1 and β2, with β1 necessary for hIL-12 signaling and activity (49). It is thought that IL-12 interacts with the hIL-12R β1 primarily through domains on the p40 subunit (50).

Given the need for an accessible p40 subunit, we chose to fuse the scIL-12 to the amino terminus of the H chain. We were concerned that if we fused the mIL-12.p40.linker.Dp35 to the carboxyl terminus of the Ab H chain, we would constrain the p40 subunit and lose IL-12 activity. In previous studies, it was found that both nerve growth factor (51) and B7.1 (36) had to be joined to the amino terminus of the Ab to maintain their activity in Ab fusion proteins; fusion at the carboxyl terminus of the H chain resulted in impaired activity in both cases. We find the IL-12 in our Ab-IL-12
fusion protein to be fully functional with IL-12 bioactivity comparable to rIL-12.

Our studies contrast with the work of Gilles et al., who fused the p35 subunit to the carboxyl terminus of the H chain and expressed the p40 subunit from a separate vector (32). While this approach led to the production of functional fusion proteins, the IL-12 had only one-half of the expected bioactivity. In contrast to the single-chain approach, this approach requires the separate transfection of the two IL-12 subunits and does not guarantee that they are present in equimolar concentrations. Although the p40 subunit was not fused to the Ab in Ab-IL-12 fusion protein produced by Gilles et al., the fusion of the p35 subunit to the carboxyl terminus of the Ab without any type of flexible linker may make the p40 subunit somewhat less accessible for receptor binding; this could explain the 2-fold lower IL-12 activity they observed.

Both scIL-12 (75 kDa) and H chain (60 kDa) are large. However, by providing a flexible linker between the two polypeptides, we were able to maintain the activity of both. The presence of IL-12 at the amino terminus of the V_{H} region does not sterically hinder the ability of the combining site of the Ab to interact with Ag on the cell surface and remain bound (see Figs. 3 and 4). Similarly, the IL-12 in the fusion protein appears to be unaffected in its ability to bind the IL-12R and exhibit IL-12-mediated cellular activation (Figs. 5–7). The attachment of the IL-12 to the V region of the Ab should position it near the surface of the tumor cell and may further potentiate the antigenicity of the targeted tumor.

The ultimate goal of the construction of mscIL-12.her2.IgG3 is its use as an antitumor agent. Using a CT26/Her2 tumor model previously developed in our laboratory (33), our initial in vivo studies demonstrate that this fusion protein has significant antitumor activity in immunocompetent BALB/c mice (Fig. 8). We observed better antitumor activity when treatment was started after the tumors were established with a mean diameter of 8–9 mm than when treatment was started the day after inoculation with tumor cells. This lends support to previous studies by others (4, 52, 53) in which better antitumor activity of IL-12 was observed when tumors were established. They proposed that this may be because effector cells are first recruited to the tumor site and are then activated by IL-12. Further work is being conducted to determine whether the in vivo efficacy we have observed is due to activated T or NK cells, whether a Th1 response has been stimulated, and whether any other antitumor activities may have been stimulated by treatment with mscIL-12.her2.IgG3.

In conclusion, we have demonstrated that it is possible to genetically engineer and express a scIL-12-Ab fusion protein that retains Her2 neu Ag specificity and IL-12 biologic activity comparable to rIL-12. Our results indicate that the bulky size of IL-12 does not affect Ag binding and that the Ab does not hinder cytokine receptor binding. Further, this fusion protein demonstrates
antimurine activity in a tumor model using CT26/Her2 cells in syngeneic immunocompetent BALB/c mice. Thus, this Ab-IL-12 fusion protein may be an effective alternate to systemic administration of IL-12 for the treatment of metastatic breast cancer. Using the tumor-targeting ability of the Ab, it should be able to achieve effective local IL-12 concentration at the sites of tumors and metastases with lower doses of IL-12, thus decreasing the risk of toxicity associated with IL-12 treatment. An anti-Her2/new mAb has had success in clinical trials for the treatment of Her2/new-expressing metastatic breast cancer (20). Fusion of a cytokine-like IL-12 that has antimurine and antimetastatic properties to a Her2/new-specific Ab may enhance its efficacy, particularly if it elicits a tumor-specific immune response.

Acknowledgments

We thank Richard Mulligan of Harvard Medical School for providing mcsIL-12 CDNA. Stanley Wolfe of Genetics Institute for providing the mIl-12 reference standard. Paul Carter of Genentech for providing the sequences of humanized huabD4S Ab. Chiron for providing rH-L2, and Jim Johnston and Thi-Sao Migne of DNAX Research Institute for their helpful suggestions.

References

SINGLE-CHAIN IL-12-ANTIBODY FUSION PROTEIN


Mechanism of Anti-tumor Activity of a Single-Chain IL-12 IgG3 Antibody Fusion Protein (mscIL-12.her2.IgG3)$^1$.

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**Running title:** Mechanism of mscIL-12.her2.IgG3 anti-tumor activity

**Keywords:** antibody fusion proteins, interleukin-12, cytokines, immunomodulators, immunotherapy.
Abstract

We have constructed an antibody-(IL-12) fusion protein (mscIL-12.her2.IgG3) that demonstrates significant anti-tumor activity against the murine carcinoma CT26 expressing human HER2/neu. We now report that this anti-tumor activity is dose-dependent and comparable or better than recombinant murine IL-12 using subcutaneous and metastatic models of disease. The anti-tumor activity of mscIL-12.her2.IgG3 is reduced in Rag2 knockout mice, suggesting that T cells play a role in tumor rejection. In SCID-beige mice, the anti-tumor activity is further reduced, suggesting that NK cells and/or macrophages are also important. The isotype of the antibody response to HER2/neu is consistent with a switch from a Th2 to a Th1 immune response and the infiltration of mononuclear cell in tumors from mice treated with mscIL-12.her2.IgG3. Some anti-tumor activity was also observed against CT26 tumors not expressing HER2/neu suggesting that mscIL-12.her2.IgG3 may also act through a non-antigen-specific mechanism. Immunohistochemistry reveals that mscIL-12.her2.IgG3 is anti-angiogenic. Thus, the mechanism of the anti-tumor activity exhibited by mscIL-12.her2.IgG3 is highly complex and involves a combination of T and NK cell activity, a switch to a Th1 immune response and anti-angiogenic activity. These studies suggest that antibody-(IL-12) fusion protein will be useful for the treatment of human cancer.
Introduction

Interleukin-12 (IL-12), a heterodimeric cytokine released by professional antigen-presenting cells, promotes cell-mediated immunity by inducing naive CD4+ T cells to differentiate into Th1 cells (1-3). In addition, IL-12 has the ability to enhance the cytotoxicity of NK and CD8+ T cells (2, 4). Moreover, the IFN-γ produced by IL-12 stimulated T and NK cells can retard tumor growth by eliciting an inhibition of tumor angiogenesis (2, 5, 6) and enhancing immune recognition of tumor cells through up-regulated MHC expression (7). IL-12 does not have a direct anti-proliferative effect on tumor cells *in vitro*, though it may inhibit tumor cell attachment to matrices and growth factor-induced invasion (8).

Systemic administration of IL-12 to mice bearing s.c. tumors results in dose-dependent tumor growth inhibition, prolongation of survival, and even tumor regression in some models (8-10). Treatment with IL-12 has been demonstrated to inhibit established experimental pulmonary and hepatic metastases and to reduce spontaneous metastases (8-11). Further, clinical trials using IL-12 in patients with cutaneous T cell lymphoma, renal cell carcinoma, and melanoma have demonstrated efficacy (12-15). However, the systemic administration of recombinant IL-12 in humans has been limited by severe toxicity making it impossible to achieve an effective dose at the site of the tumor (2, 13, 16). Ideally, strategies that increase the cytokine concentration at the site of the tumor and allow for lower systemic levels should be more effective.

Antibody-(IL-12) fusion proteins in which tumor-specific antibodies can be used to selectively target IL-12 to tumors provide an attractive delivery vehicle. The specific targeting should make it possible to achieve effective doses at the site of the tumor without accompanying systemic toxicity. However, IL-12 is a disulfide-linked heterodimer of two subunits p35 and p40
and requires the expression of two separate genes and correct heterodimer assembly for activity (17). To address this issue, Gillies et al. constructed an antibody-(IL-12) fusion protein specific for the pan-carcinoma antigen EpCAM, in which the p35 subunit was fused to the carboxy terminus of a human IgG1 heavy chain; the p40 subunit was expressed as a separate polypeptide which must then assemble with the p35 subunit (18). A construct composed of murine p35 and human p40 proved to be highly effective at treating SCID mice with established pulmonary metastasis of the CT26 carcinoma expressing human EpCAM (18), despite the fact that this antibody-(IL-12) fusion protein was less active than murine IL-12.

As an alternative approach, we have constructed an antibody-(IL-12) fusion protein, mscIL-12.HER2.IgG3, in which single-chain murine IL-12 (mscIL-12) was joined to the amino terminus of an IgG3 antibody by a flexible Gly-Ser linker (19). The IL-12 in this fusion protein has been modified in that the two IL-12 subunits are covalently linked and this single-chain IL-12 is further tethered to a large antibody molecule making it of interest to determine the mechanisms of the anti-tumor activity of the IL-12 in the fusion protein (19). The mscIL-12.HER2.IgG3 contains the variable region of trastuzumab (Herceptin, Genentech, San Francisco, CA) specific for the extracellular domain of HER2/neu (ECD<sup>HER2</sup>) (20), a tumor associated antigen (TAA) the over-expression of which is associated with poor prognosis in many different cancers including breast, ovarian, lung, and gastric (21-24). Although treatment of HER2/neu expressing tumors with trastuzumab has shown some efficacy (25, 26) improved therapies are still needed for the treatment of HER2/neu expressing tumors.

Although our long-term goal was to improve the anti-tumor effect of trastuzumab by the production of an antibody-(IL.12) fusion proteins for therapeutic use in humans, murine IL-12 was used for these initial studies because it has activity on both human and murine cells, while
human IL12 has activity only on human cells (19). The use of murine IL-12 makes it possible to
both test biologic activity using human PBMC and to perform in vivo studies to examine the
effects against human HER2/neu-expressing murine tumors using immune-competent mice. We
found that mscIL-12.her2.IgG3 retains antigen specificity, and IL-12 bioactivity in vitro and
demonstrates anti-tumor activity in BALB/c bearing s.c. CT26-HER2/neu tumors under
conditions in which treatment with anti-HER2/neu IgG3 failed to confer protection. Since the
ultimate goal is to use mscIL-12.her2.IgG3 as an anti-tumor agent and the mechanism of IL-12
anti-tumor activity appears to depend upon many variables including the dosing regimen, it was
important to determine the mechanism of the anti-tumor activity of the antibody fusion proteins
and compare it with that of IL-12. It should be noted that in our previous studies (19) and in the
report of Gillies et al. (18) a control of free IL-12 was not included in the animal experiments
making it impossible to determine if the antibody-(IL-12) fusion protein differs in activity from
free IL-12.

In the present studies we expanded the characterization of mscIL-12.her2.IgG3 by
studying its anti-tumor activity in immune-competent BALB/c using CT26-HER2/neu models
previously developed in our laboratory (27). In addition, we directly compared the activity of
mscIL-12.her2.IgG3 to that of equivalent doses of free recombinant murine IL-12 (rmIL-12). We
show that anti-tumor activity of mscIL-12.her2.IgG3 is highly complex and reflects among other
things a switch to a Th1 response, tumor infiltration by T and NK cells, and anti-angiogenic
activity.
Materials and Methods

Cell lines and reagent: CT26, a murine colon carcinoma induced in BALB/c mice by intrarectal injection of N-nitroso-N-methylurethane (28), was kindly provided by Dr. Young Chul Sung (Pohang University of Science and Technology, Korea). CT26-HER2/neu cells were developed in our laboratory by transduction of CT26 cells with the cDNA for HER2/neu (27). Both cell lines were cultured in IMDM (Irvine Scientific Inc., Irvine, CA) supplemented with 5% bovine calf serum (Atlanta Biologicals, Norcross GA) at 37°C with 5% CO₂. mscIL-12.her2.IgG3 was purified from culture supernatants as previously described (19). Murine recombinant IL-12 was kindly provided by Dr. Stanley Wolf (Genetics Institute, Cambridge, MA).

Mice. Female 6-8 week old BALB/c and SCID-beige (C.B-17 SCID-beige) mice were obtained from Taconic Farms, Inc. (Germantown, NY). Female 6-8 week old Rag2<sup>−/−</sup> knockout mice (129 Rag2) were kindly provided by Colin McLean at UCLA. All mice were conventionally housed and all animal experiments were approved by the UCLA Review Board of Animal Research Committee.

Experimental metastatic model and anti-tumor treatment. On day 0 of the experiment, female 6-8 week old BALB/c mice (8 mice per treatment arm) were injected i.v. with a single cell suspension of 5x10⁴ CT26-HER2/neu cells resuspended in 0.3 ml of HBSS (GibcoBRL, Grand Island, NY) to induce pulmonary metastases. Mice were mixed, randomly segregated into the indicated groups, and on days 3-7 of the experiment treated with daily i.v. injections of PBS or PBS containing 1 μg IL-12 equivalent of mscIL-12.her2.IgG3 or 1 μg of rmIL-12. On day 16,
each mouse was euthanized, the lungs removed and the number of metastases determined using the procedure of Wexler et al. with modifications (29). The trachea was exposed, a small incision made, and India ink (15%) was slowly pushed into the lungs through a needle and syringe inserted into the trachea. The lungs were removed and placed in water. The lungs were blotted on a paper towel and placed in Bouin’s fixative solution (Ricca Chemical Co., Arlington, TX) overnight. The lungs were washed 3x with 70% ethanol. The number of metastases was counted under a dissecting microscope. A control of anti-HER2/neu IgG3 was not included in this experiment because we have previously observed that lung metastases of CT26-HER2/neu cells are resistant to the treatment with antibody alone (anti-HER2/neu IgG3) at the dose greater than that used in the present study (Peng et al., unpublished results).

Subcutaneous tumor model and anti-tumor treatment. Female 6-8 week old BALB/c, Rag2 knockout, or SCID-beige mice (6-8 mice per treatment arm) were injected s.c. in the right flank with a single cell suspension of 1x10^6 CT26 or CT26-HER2/neu cells resuspended in 0.15 ml of Hank’s Balanced Salt Solution (HBSS) (GibcoBRL) on day 0 of the experiment. The mice were mixed, segregated randomly into the indicated groups and on days 6-10 of the experiment were treated with daily i.v. injections of PBS or PBS containing 1 or 5 µg IL-12 equivalent of mscIL-12.1G3 or 1 or 5 µg of rmIL-12. The tumor size was monitored by caliper measurement every other day and tumor volume calculated. On day 20-22 mice were euthanized and tumor and sera harvested for subsequent studies. Tumors were processed for histological and immunohistochemical analysis. Blood samples were collected, serum was separated from clotted blood and stored at -20°C until assayed by ELISA. The control of anti-HER2/neu IgG3 was not
included in these experiments because we have previously observed that s.c. CT26-HER2\textit{neu} tumors are resistant to the treatment with anti-HER2\textit{neu} IgG3 (19).

\textit{Tumor histology}. Immediately after harvest, tumors were frozen in OCT embedding compound (Miles Inc., Elkhart, IN). Tumor sections were cut to 6 µm and fixed in 10% formalin, washed, then immersed in hematoxylin aqueous formula (Biomedica, Foster City, CA) for 5 min. The slides were washed and immersed in Scott’s water (Fisher Chemical, Somerville, NJ) for 1 min and then washed, immersed in Eosin Y solution alcoholic with phloxine (Sigma Chemical, St. Louis, MO) for 1 min and 15 seconds. The slides were then dehydrated in ethanol, and cleared in xylene, and mounted with Permount (Fisher Chemical).

\textit{Time course of early response in mice bearing s.c. CT26-HER2\textit{neu} tumors}. On day 0 of the experiment, 6-8 week old female BALB/c mice were injected with $1 \times 10^6$ CT26-HER2\textit{neu} cells/mouse s.c. in the right flank. Twenty-six mice were used in the experiment with 2 mice in the pretreatment and PBS-treated groups and 3 mice in all other treatment groups. The mice were treated with PBS or 1 µg IL-12 equivalents/day of mscIL-12.her2.IgG3 or rmiIL-12 at for 5 days beginning on day 6 after tumor inoculation. The mice were not treated if they were to be sacrificed that day. The mice were sacrificed at four different time points: 24 h before treatment was initiated (pretreatment group) and 24 h, 4 days, and 7 days after treatment was initiated. At each time point, blood was collected and the tumor and liver harvested.

\textit{Anti-CD3/anti-NK1.1 cell staining}. To determine the phenotype of the lymphocytes present in tumor-bearing animals, 0.5-1x10$^6$ lymphocytes isolated from the livers were added to 2 ml of
PBS mix (PBS + 0.1% NaN₃ + 2% bovine calf serum). The tubes were centrifuged for 5 min, 180 g, at 4°C, decanted and drained on 3 mm Whatman filter paper. The cells were incubated with 100 μL of hamster anti-mouse CD3 and biotinylated anti-mouse NK1.1 for 2 h at 4°C with agitation. All antibodies were used at a concentration of 1 μg/100 μl in PBS mix and were purchased from Pharmingen (San Diego, CA). A hamster IgG isotype matched non-specific antibody and PBS mix were used as controls for the anti-CD3 and the anti-NK1.1 antibodies, respectively. After 2 ml PBS mix was added, each sample was centrifuged for 5 min, 180 g, at 4°C, decanted, drained on filter paper and washed with another 2 ml PBS mix. The samples were then incubated in diluted FITC-conjugated anti-hamster antibody and diluted streptavidin-PE at 0.5 μg/1500 μl (Pharmingen,) for 45 min at 4°C with agitation. Two ml PBS mix was added to each sample. They were then centrifuged for 5 min, 180 g, at 4°C, decanted and drained on filter paper. 200 μl 2% paraformaldehyde was added to each sample to fix the cells and the samples were stored covered at 4°C until they could be analyzed by flow cytometry. Analysis was performed with a FACScan (Becton Dickinson, Mountain View, CA) equipped with a blue laser excitation of 15 mW at 488 nm. The two fluorochromes, FITC and PE, were electronically compensated using singly-labeled spleen cells.

**Anti-HER2/neu ELISA.** Immulon 2 96-well plates (Dynex Technologies Inc., Chantilly, VA) were coated (50 μl/well) with ECDHER2 (1 μg/ml) in carbonate buffer pH 9.6, and incubated at 4°C overnight. The plates were washed 5x with PBS and blocked overnight at 4°C with 100 μl/well of 3% BSA in PBS + 0.02% NaN₃. The plates were washed 5x with PBS. Serum (pooled from a treatment group, diluted 1:50 in 1% BSA in PBS) was added at 50 μl/well in triplicate. The plates were incubated overnight at 4°C. The plates were washed 5x with PBS. Rat
antibodies specific for murine IgG1 and IgG2a, (Pharmingen) were diluted 1:5 in 1% BSA in PBS and added at 50 µl/well to the plates. The plates were incubated at room temperature for 2 h. The plates were washed 5x with PBS. Goat-anti-rat antibody diluted 1:1000 in 1% BSA in PBS conjugated to alkaline phosphatase (Pharmingen) was added at 50 µl/well and the plates incubated at 37°C for 1 h. The plates were washed 5x with PBS, 50 µl/well phosphatase substrate (1 tablet p-nitrophenyl phosphate (Sigma Chemical) per 5 ml diethanolamine buffer (49 ml diethanolamine + 120 µl 1 M mgCl2 + dH2O to 500 ml, pH 9.8) added, and the plates were read at 410 nm after developing 1-2.5 h at room temperature.

Anti-angiogenic activity. Immediately after harvest, tumors were frozen in OCT embedding compound (Miles Inc., Elkhart, IN). Tumor sections were cut to 6 µm and fixed in acetone. The slides were rehydrated in PBS pH 7.5 (3 changes in 5 min). Any endogenous peroxidase activity was quenched by immersing the slides in 0.3% H2O2 in methanol for 30 min. The slides were washed in PBS pH 7.5 (3 changes in 5 min). The sections were blocked in blocking buffer (3% BSA in PBS pH 7.5) for 20 min in a humidified chamber. The blocking buffer was aspirated off the slides and rat antibodies to murine PE-CAM (Pharmingen), an adhesion molecule (CD31) on endothelial cells, diluted to 0.5 µg/ml in blocking buffer were added to the sections. After overnight incubation in a humidified chamber at room temperature, the slides were washed in PBS pH 7.5 (3 changes in 5 min). Diluted biotinylated anti-rat antibody, mouse adsorbed (Vector Labs, Burlingame, CA), was added to the sections at 2.5 µg/ml. After 1 h incubation in a humidified chamber at room temperature, the slides were washed in PBS pH 7.5 (3 changes in 5 min). Vectastain Elite ABC (Vector Labs; 2 drops A and 2 drops B per 5 ml PBS pH 7.5 mixed 30 min before use) was added to the slides and they were placed in a humidified chamber for 30
min at room temperature. The slides were washed in PBS (3 changes in 5 min), SG substrate (Vector Labs; 3 drops chromagen and 3 drops H₂O₂ solution per 5 ml PBS pH 7.5) added and allowed to develop for 10 min. The slides were washed in PBS pH 7.5 (3 changes in 5 min) followed by a short dip in dH₂O. Nuclear fast red counterstain (Vector Labs) was added to the sections and allowed to develop for 10 min. After washing in 3 changes of tap water, the slides were dehydrated in successive incubations in 95% ethanol (3x, 2 min each), and 100% ethanol (3x, 2 min each). The slides were then cleared in 7 changes of xylene and mounted with Permount (Fisher Chemical).

Photographs of the sections (at least 3 fields per tumor section, average 5) were taken and the photographs were overlaid with a 5x5 μm grid and vessel intersections counted. The number of intersections per view was averaged for all tumors within the same treatment group at the same time point and standard deviations calculated.

*Statistical analysis.* Statistical analysis of the anti-tumor experiments was done using a two-tailed Student’s t-test. Results were regarded significant if P values were ≤ 0.05.

Results

*Anti-tumor activity of rmIL-12 and mscIL-12.her2.IgG3 in BALB/c mice bearing CT26-HER2/neu pulmonary metastases.* Since one of the potential applications of an antibody fusion protein would be to target metastatic disease, we examined the anti-tumor activity of mscIL-12.her2.IgG3 in an experimental CT26-/HER2/neu lung metastasis model. Fig. 1 shows that treatment of mice with rmIL-12 or the equivalent IL-12 amount of mscIL-12.her2.IgG3 resulted
in a potent inhibition of in the number of CT26-HER2/neu pulmonary metastases (mean number of metastases 20 and 22, respectively) as compared with PBS-treated controls (mean: 88). In both cases this difference was statistically significant $P = 0.019$ for rmIL-12 and $P = 0.025$ for mscIL-12.her2.IgG3. Although there was no significant difference between rmIL-12 and mscIL-12.her2.IgG3 we observed that while the response of mice to the treatment with rmIL-12 was rather homogeneous animals treated with mscIL-12.her2.IgG3 are split in two subgroups, one subgroup showing fewer metastases than the best responders of the group treated with rmIL-12, while the other subgroup shows more pulmonary metastases than the mice treated with rmIL-12.

*Dose-dependent anti-tumor activity of rmIL-12 and mscIL-12.her2.IgG3 in BALB/c mice bearing subcutaneous CT26-HER2/neu tumors.* In our previous experiments using mscIL-12.her2.IgG3 at 1 μg/day x 5 days, we observed tumor growth arrest when the fusion protein was administered on days 6-10 after tumor inoculation inoculation. We now examined the anti-tumor activity of mscIL-12.her2.IgG3 and rmIL-12 at 1 μg/day and a higher dose (5 μg/day) for 5 days. These experiments were designed both to compare the efficacy of the fusion protein with mIL-12 and to determine whether we could obtain complete tumor regression and whether any treatment-limiting toxicity could be observed at the higher dose. We found that treatment of mice with rmIL-12 or the equivalent IL-12 amount of mscIL-12.her2.IgG3 resulted in a potent inhibition of s.c. CT26-HER2/neu tumor growth when compared with the group injected with PBS, with more effective inhibition seen in the mice treated with the higher doses (Fig. 2). Although similarly significant ($P < 0.0001$) dose-dependent inhibition of tumor growth was seen for both treatment groups after day 15, significant anti-tumor activity was observed at an earlier time with mscIL-12.her2.IgG3 than with rmIL-12. At days 7 and 9 mice injected with 5 μg rmIL-12 equivalent of
mscIL-12.her2.IgG3 showed a significant inhibition of tumor growth ($P=0.007$ and $P=0.004$ respectively) that was not observed in the group injected with 5 μg rmIL-12 ($P=0.5$ and $P=0.4$ respectively). Similarly, mice injected with 1 μg rmIL-12 equivalent of mscIL-12.her2.IgG3 showed a significant inhibition of tumor growth by days 11 and 13 ($P=0.02$ for both days) while significant inhibition was not observed at that time in the group injected with 1 μg rmIL-12 ($P=0.16$ and $P=0.18$ respectively). Complete tumor regression was not observed using either rmIL-12 or mscIL-12.her2.IgG3 with these treatment regimens. Although the average volumes of the tumors in the two treatments groups were similar, as we had observed in the treatment of metastatic disease (Fig 1), the response of mice to the treatment with rmIL-12 was rather homogeneous while animals treated with mscIL-12.her2.IgG3 split into two groups. One group showed very potent inhibition of s.c. tumor growth while the other group had larger tumors than the mice treated with rmIL-12 (data not shown).

Examination of hematoxylin/eosin stained histologic sections of tumors obtained from mice 19 days following injection of PBS (Fig. 3, Panel A), mscIL-12.her2.IgG3 (equivalent to 1 μg IL-12) (Fig. 3, Panel B), or 1 μg rmIL-12 (Fig. 3, Panel C) showed that tumors from mice treated with PBS are highly cellular while tumors from mice treated with mscIL-12.her2.IgG3 or rmIL-12 show mononuclear cell infiltration and loss of structure. Although Fig. 3 shows the tumor histology of only one mouse per each treatment group, similar results were observed in the other members of each treatment group (data not shown).

**Anti-tumor activity of rmIL-12 and mscIL-12.her2.IgG3 in Rag2 knockout mice bearing subcutaneous CT26-HER2/neu tumors.** Since IL-12 anti-tumor activity has been attributed to T cells, we studied the activity of mscIL-12.her2.IgG3 in Rag2 knockout mice. These mice have a
disruption of the recombination activating gene 2 (Rag2) and thus are unable to initiate V(D)J rearrangement and fail to generate mature B or T cells (30). The anti-tumor activity of rmIL-12 and mscIL-12.her2.IgG3 was markedly diminished (as compared to immune-competent mice) but not completely abrogated in the Rag2 knockout mice (Fig. 4). In the case of mice injected with mscIL-12.her2.IgG3, statistically significant inhibition of tumor growth was seen on days 14, 16, 20, and 22 ($P \leq 0.04$) as compared with the group treated with PBS. Although inhibition of tumor growth was observed in mice injected with rmIL-12, it did not achieve statistical significance compared to the PBS treatment group. No mononuclear cell infiltration was observed in hematoxylin/eosin stained sections of tumors obtained from Rag2 mice following injection of PBS, mscIL-12.her2.IgG3 or rmIL-12 (data not shown).

**Anti-tumor activity of rmIL-12 and mscIL-12.her2.IgG3 in SCID-beige mice bearing subcutaneous CT26-HER2/neu tumors.** To further identify the immune cells responsible for the anti-tumor activity of mscIL-12.her2.IgG3, we studied the efficacy of the fusion protein in SCID-beige mice. The SCID (severe combined immunodeficient) mutation causes a defect in V(D)J recombination leading to a deficiency in B and T cells. The beige mutation results in impaired chemotaxis and motility of macrophages and a deficiency of NK cells (31). Therefore these mice should indicate if mscIL-12.her2.IgG3 and rmIL-12 have any anti-tumor activity in mice deficient in T- and NK cells and in macrophage activity. Fig. 5 shows that the anti-tumor activity of rmIL-12 and mscIL-12.her2.IgG3 was markedly diminished but not completely abrogated in the SCID-beige mice. Similar inhibition of tumor growth was seen in mice injected with mscIL-12.her2.IgG3 or rmIL-2. By days 16 and 18 this inhibition was statistically significant ($P < 0.05$) compared to the group treated with PBS. Both mscIL-12.her2.IgG3 and
rmIL-12 demonstrated somewhat less anti-tumor activity in the SCID-beige mice than in the Rag2 knockout mice. The average tumor volume of mscIL-12.her2.IgG3 treated Rag2 knockout mice at day 20 was 1906 mm$^3$ ± 581, while in the SCID-beige mice it was 2381 mm$^3$ ± 804. The Rag2 knockout and SCID-beige mice treated with PBS had similar average tumor volumes of 3695 ± 1513 and 3772 ± 710 mm$^3$, respectively.

*Anti-tumor activity of rmIL-12 and mscIL-12.her2.IgG3 in BALB/c mice bearing subcutaneous CT26 tumors.* Our studies have indicated that mIL-12 and mscIL-12.her2.IgG3 have similar anti-tumor activity against CT26-HER2/neu. To explore the contribution of antigen targeting to efficacy, we examined the effect of mIL-12 and mscIL-12.her2.IgG3 treatment BALB/c mice bearing CT26 tumors that do not express HER2/neu (Fig. 6). In this system both mscIL-12.her2.IgG3 and rmIL-12 demonstrated anti-tumor activity although the anti-tumor effect was not as great as that observed with the CT26-HER2/neu tumors. When PBS treated controls had average tumor volumes of 2500 mm$^3$, the rmIL-12 treated mice had tumor volumes of 1845 and 650 mm$^3$ and the mscIL-12.her2.IgG3 treated mice had tumors of 2387 and 650 mm$^3$ for CT26 and CT26-HER2/neu respectively. On days 16 and 18, the inhibition of tumor growth was statistically significant ($P< 0.02$) for both mscIL-12.her2.IgG3 and rmIL-12. Although rmIL-12 appears to have a greater anti-tumor effect than mscIL-12.her2.IgG3, this difference is not statistically significant.

*Distribution of CD3+ and NK1.1+ cells in the liver of tumor bearing animals.* To further understand the mechanism of the anti-tumor activity exhibited by mscIL-12.her2.IgG3, we examined mice bearing tumors at various times following treatment. On day 0, CT26-HER2/neu
cells were injected s.c. into the right flank of BALB/c mice. The mice were treated with mscIL-12.her2.IgG3, rmIL-12, or PBS for five days beginning on day 6. Mice were sacrificed before the initiation of treatment (day 5) or 24 h (day 7), 4 days (day 10), and 7 days after treatment was initiated (day 13). Lymphocytes isolated from the livers of the mice were double-stained for NK cells (NK1.1) and T cells (CD3) and analyzed by flow cytometry. A representative flow cytometry profile of lymphocytes isolated from the liver at day 7 is shown in Fig. 7 (Panel A) with the regions gated representing CD3+ (CD3+/NK1.1-), NK1.1+ (CD3-/NK1.1+) and double-positive (CD3+/NK1.1+) cells. The distribution of the CD3+, NK1.1+ and double-positive cells for each treatment group is shown in Fig. 7 (Panel B).

Although all three treatment groups showed a similar decrease in the percentage of lymphocytes staining with CD3 and/or NK1.1+ over time, there were differences among the groups in the percentage of CD3+, NK1.1+ and double-positive cells. Treatment with rmIL-12 and mscIL-12.her2.IgG3 resulted in an increase in the percentage of NK1.1+ cells that was clearly seen on days 10 and 13. The increase was seen in both CD3+/NK1.1+ and CD3-/NK1.1+ cells. These results demonstrate that both mscIL-12.her2.IgG3 and rmIL-12 are able to comparably increase the number of NK1.1+ cells in the liver. Especially noteworthy was the increase in CD3+/NK1.1+ cells seen on day 10 in the mscIL-12.her2.IgG3 treatment group.

*Isotype of the HER2/neu specific antibody response.* The ability of IL-12 to switch the immune response to Th1 has been implicated in its anti-tumor activity. We previously reported that an anti-HER2/neu response was seen in our CT26-HER2/neu tumor model (27). However, this response does not appear to affect tumor growth or to select for non-HER2/neu-expressing tumor cells (27) or for loss of expression of MHC class I (Penichet et al., unpublished results) in the
tumor mass. To examine the isotypes of the HER2/\textit{neu} specific antibody response in BALB/c mice bearing CT26-HER2/\textit{neu} tumors treated with PBS, rmIL-12, and mscIL-12.her2.IgG3 (described in Fig.1), serum collected on day 20 (approximately 14 days after treatment was initiated on day 6) from each treatment group was pooled and used in an anti-HER2/\textit{neu} ELISA (Fig. 8). Mice treated with mscIL-12.her2.IgG3 or rmIL-12 exhibited a decrease in IgG1 (Th2) HER2/\textit{neu} specific antibodies relative to PBS-treated controls. Variability is seen in the effects of rmIL-12 and mscIL-12.her2.IgG3 on the levels of IgG2a (Th1) HER2/\textit{neu} specific antibodies but in general we observed an increase in the IgG2a immune response in mice treated with free rmIL-12 or IL-12 fused to IgG3 (mscIL-12.her2.IgG3). Similar results were obtained within a week of treatment on day 13 (data not shown).

\textit{Anti-angiogenic activity}. Our studies have demonstrated that mscIL-12.her2.IgG3 has anti-tumor activity that is attributable to T and NK cells. However, our data also suggest that additional mechanisms may be involved. To examine whether mscIL-12.her2.IgG3 has any anti-angiogenic activity, tumor sections from various times during treatment were stained for PE-CAM, an adhesion molecule (CD31) on endothelial cells (Fig. 9, Panel A). Fields that spanned the entire area of the tumor section were photographed, grid overlaid on the photographs and vessel intersections counted (Fig. 9, Panel B). A decrease in vessel count was seen 4 and 7 days after treatment with mscIL-12.her2.IgG3 and rmIL-12 was initiated. These results demonstrate that both mscIL-12.her2.IgG3 and rmIL-12 have anti-angiogenic activity.
Discussion

We previously described the construction of an IL-12-anti-HER-2/neu antibody fusion protein (mscIL-12.her2.IgG3) that retains antigen specificity and IL-12 bioactivity in vitro and demonstrates anti-tumor activity (19). In these studies, we further characterize this anti-tumor activity in CT26-HER2/neu models (27) of s.c. and metastatic disease and we describe the mechanism of this anti-tumor activity.

For these studies we have used CT26 (also known as C-26 and Colon Tumor 26), a murine colon carcinoma (epithelial origin) syngeneic to BALB/c, that was induced by intrarectal injection of N-nitroso-N-methylurethan (28). The CT26 tumor is a relevant model for testing immunotherapeutic approaches to cancer treatment because human cancers of epithelial origin are among the most difficult to treat by existing immunotherapies(32, 33). However, variable results have been reported following systemic IL-12 treatment of mice bearing CT26 tumors. While there are reports that s.c. CT26 tumors growing in immune-competent mice are refractory to systemic treatment with IL-12 (34, 35), other authors such as Tannanbaum et al. have reported that systemic treatment with IL-12 is highly effective (11). Our studies have shown that both CT26-HER2/neu and the parental CT26 tumors are sensitive to both rmIL-12 and mscIL-12.her2.IgG3 highlighting the importance of using free rmIL-12 as a control when evaluating fusion protein efficacy. However, we observed that CT26-HER2/neu responds much better to the therapy with both rmIL-12 and mscIL-12.her2.IgG3 than the parental CT26 tumor. Therefore, it is possible that expression of the HER2/neu antigen altered the sensitivity of the CT26 cells to IL-12 or made the tumor cells more readily recognized by immune effector cells.

Although direct comparisons between the CT26 and CT26-HER2/neu results are not possible, our results are consistent with the study of Tannanbaum et al. which showed that CT26
responded to systemic treatment with IL-12 (11). In that study systemic treatment with IL-12 lead to IFN-γ and IP-10 production (11) that could be detected as early as 4 h after IL-12 administration and became maximal at approximately 6 days with continued IL-12 treatment. Further, immunohistologic analysis revealed infiltration with CD8+ T cells and Mac-1+ mononuclear cells (Mac-1 is found on NK cells and macrophages), but very low or negligible infiltration by CD4+ T cells (11). Tumor regression was associated with expression of perforin and granzyme B, consistent with the hypothesis that one mechanism by which cytolytic T and NK cells mediate cytolysis of tumor targets is by exocytosis of effector molecules stored in granules (11).

Earlier studies had shown that treatment of SCID mice bearing pulmonary metastases of CT26 expressing human EpCAM with an anti-EpCAM antibody-(IL-12) fusion protein results in a significant anti-tumor activity although complete eradication of tumors was not achieved (18). We now demonstrate using immune-competent BALB/c instead of SCID mice that mscIL-12.her2.IgG3 is highly effective in inhibiting the number of CT26-HER2/neu pulmonary metastases. We also found that treatment of mice with free rmIL-12 results an anti-tumor activity similar to mscIL-12.her2.IgG3; the control of free IL-12 was not used in the earlier studies (18).

We have also found that treatment with rmIL-12 or the mscIL-12.her2.IgG3 results in a potent anti-tumor activity against s.c. CT26-HER2/neu tumors with efficacy increasing at higher doses. This is consistent with previous reports showing that the anti-tumor effect of IL-12 is dose-dependent and can be initiated against well-established tumors (8). Tumor histology showed loss of structure and fragmentation associated with mononuclear cell infiltration, presumably T-cells since no infiltration is seen in Rag2 deficient mice following similar treatments. T-cell infiltration is consistent with the ability of IL-12 to elicit a CTL immune
response (2, 4, 8). In fact, in patients receiving IL-12, tumor biopsy and immunohistochemistry revealed CD4+ and CD8+ T cell infiltration (14, 15).

The fact that similar anti-tumor activity is seen following treatment of both pulmonary and s.c. CT26-HER2/neu tumors with mscIL-12.her2.IgG3 and rmIL-12 suggests that tumor targeting does not improve the anti-tumor response following treatment. However, mscIL-12.her2.IgG3 appeared more effective than rmIL-12 at early times during treatment (Fig 2). In addition, mscIL-12.her2.IgG3 was less effective than rmIL-12 against CT26 that did not express the targeting antigen (although the difference did not reach statistical significance) suggesting that tumor targeting may play a role in the anti-tumor mechanism of mscIL-12.her2.IgG3. It is noteworthy that other investigators were unable to perform studies with IL-12 fusion proteins in immune-competent mice because the fusion protein was too immunogenic (18). Although we do see some efficacy of the fusion proteins in our studies, it is possible that the fused murine IL-12 acts as an adjuvant and increases the murine humoral immune response against human immunoglobulin sequences of the antibody-(IL-12) fusion protein resulting in its neutralization and as a consequence, in less effective anti-tumor activity. Similar enhancement of a murine anti-human IgG humoral immune response has been described for an anti-HER2/neu IgG3-(GM-CSF) fusion protein (36). This hypothesis may explain the observation that greater variation is seen in tumor size in mice treated with mscIL-12.her2.IgG3 than in those treated with rmIL-12. It is possible that the mice with larger tumors mounted a more potent humoral immune response to mscIL-12.her2.IgG3. Further studies are required to investigate the relationship between the immunogenicity and efficacy of antibody-(IL-12) fusion proteins. However, if the murine immune response to the human immunoglobulin sequences of the antibody-(IL-12) fusion protein does limit efficacy in mouse models, this suggests that the antibody-(IL-12) fusion
protein will be much more effective in the treatment of patient, the ultimate goal of this fusion protein.

A role for T-cells in the anti-tumor activity of IL-12 is supported by our experiments using Rag2 mice. In the Rag2 mice which lack B and T cells (30), both IL-12 and mscIL-12.her2.IgG3 showed less potent anti-tumor activity than in immune-competent mice and there was a lack of mononuclear cell infiltration. It is also of interest that in these mice which cannot mount an immune response to the antibody-(IL-12) fusion protein, a statistically significant retardation in the tumor growth rate was found following treatment with mscIL-12.her2.IgG3 but not following treatment with free rmIL-12. In these mice NK and/or macrophage activation by IL-12 should be responsible for the anti-tumor activity (8, 9, 37). In fact, the strong inhibition of CT26-EpCAM pulmonary metastases observed in SCID mice following treatment with the anti-EpCAM antibody-(IL-12) fusion protein led the authors to suggest that in this model, the antibody-(IL-12) fusion protein was a potent activator of NK cells (18).

The further decrease in the anti-tumor activity of mscIL-12.her2.IgG3 that we observed in SCID-beige mice, which are not only T and B cell deficient but also have impaired chemotaxis and motility of macrophages and NK cell deficiency (31), is consistent with macrophage and/or NK cell activation contributing to the anti-tumor activity of antibody-(IL-12) fusion proteins. However, since there was some residual anti-tumor activity in the SCID-beige model, additional (non-cellular) mechanisms must also contribute to the anti-tumor activity of mscIL-12.her2.IgG3. Indeed we found that mscIL-12.her2.IgG3 and rmIL-12 demonstrated significant anti-angiogenic activity consistent with an increase in IFN-γ and subsequent increase in IP-10, an inhibitor of angiogenesis (2, 5, 6).
We found that treatment with rmIL-12 or mscIL-12.her2.IgG3 resulted in a change in the composition of the lymphocyte population present in the liver. We observed an increase in the percentage of both CD3+/NK1.1+ and double-positive (CD3+/NK1.1+) cells. This increase persisted throughout the course of the experiment (Figure 7). In contrast, Folger et al. found that IL-12 administration (0.5 µg/day i.p. for 7 days) resulted in an increase in hepatic NK1.1+ cells 24 h after IL-12 administration was initiated, but this increase was not sustained; instead CD3+ cells gradually increased during the time that IL-12 was administered (38). The differences between what we and Folger et al. observed might be explained by the different dosing regimens. IL-12 stimulates the production of IL-2; high doses of IL-2 are known to stimulate NK cell-mediated anti-tumor activity, while low doses stimulate a T cell-mediated effect (39). Different dosing regimens and routes of administration of IL-12 may lead to different levels of IL-2 induction. Use of different mouse strains may also contribute to the differences as Fogler et al. used C57BL/6 mice and we used BALB/c mice. C57BL/6 mice produce a Th1 response to infection with Leishmania major while BALB/c mice generate a Th2 response (40). Hashimoto et al. had previously demonstrated that NK1.1+/CD3+ cells are induced in the liver by IL-12 and that these cells demonstrate MHC-nonrestricted cytotoxic activity (41). Thus, the increase in NK and NK1.1+/CD3+ cells following treatment with mscIL-12.her2.IgG3 may contribute to its anti-tumor activity.

In conclusion, it would appear that the anti-tumor activity of mscIL-12.her2.IgG3 in a CT26-HER2/neu model is highly complex and involves the activity of T cells, NK cells and/or macrophages as well as anti-angiogenic effects. Therefore, in spite of the genetic modification of IL-12 in the fusion protein it maintains an activity profile similar to that of IL-12. The potent
anti-tumor activity of the anti-HER2/neu-(IL-12) fusion protein suggest that it may be of clinical utility in the treatment of patients with tumors expressing HER2/neu.
Acknowledgements

We are grateful to Dr. Donald Morrison and to Dr. Colin McLean from the University of California, Los Angeles, for assistance in the statistical analysis and for the Rag2 knockout mice respectively. We thank Dr. Stanley Wolf from Genetics Institute for providing the murine IL-12 reference standard, to Dr. Young Chul Sung from Pohang University of Science and Technology, Korea for providing the CT26 cell line, to Dr. James D. Marks from University of California, San Francisco for providing the ECD\textsuperscript{HER2}. 
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Footnotes:

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 NCI (NIH) and Cancer Center Core grant CA-16042 (UCLA). Lisan S. Peng is a student in the UCLA Medical Scientist Training Program and is supported by grant GM08042 and the Aesculapians Fund of the UCLA School of Medicine.

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Figure Legends

Figure 1. Anti-tumor activity of rmIL-12 and mscIL-12.her2.IgG3 in BALB/c mice bearing CT26-HER2/neu pulmonary metastases. BALB/c mice were injected with $5 \times 10^4$ CT26-HER2/neu cells i.v. on day 0. On days 3-7 the mice were treated with daily i.v. injection of PBS or PBS containing 1 μg IL-12 equivalent of mscIL-12.her2.IgG3 or 1 μg of rmIL-12. On day 16, the mice were euthanized, the lungs removed and the metastases counted.

Figure 2. Anti-tumor activity of different doses of rmIL-12 and mscIL-12.her2.IgG3 in BALB/c mice bearing s.c. CT26-HER2/neu tumors. BALB/c mice were injected with $1 \times 10^6$ CT26-HER2/neu cells s.c. on day 0. On days 6-10, the mice were treated with daily i.v. injection of PBS or PBS containing 1 or 5 μg IL-12 equivalent of mscIL-12.her2.IgG3 or 1 or 5 μg of rmIL-12. The tumor size was monitored by caliper measurement every other day and tumor volume calculated.

Figure 3. Histologic sections of s.c. CT26-HER2/neu tumors from BALB/c mice. Twenty day old tumors from BALB/c mice were frozen and cut in 6 μm sections. Panels A, B, and C show histologic sections of tumors from mice treated with PBS, mscIL-12.her2.IgG3, or rmIL-12 respectively. The sections were stained with hematoxylin/eosin. Bar = 100 μm.

Figure 4. Anti-tumor activity of rmIL-12 and mscIL-12.her2.IgG3 in Rag2 knockout mice bearing s.c. CT26-HER2/neu tumors. Rag2 mice were inoculated s.c. with $1 \times 10^6$ CT26-HER2/neu tumor cells on day 0. On days 6-10, the mice were treated with daily i.v. injection of
PBS or PBS containing 1 µg IL-12 equivalent of mscIL-12.her2.IgG3 or 1 µg of rmIL-12. The tumor size was monitored by caliper measurement every other day and tumor volume calculated.

**Figure 5. Anti-tumor activity of rmIL-12 and mscIL-12.her2.IgG3 in SCID-beige mice bearing s.c. CT26-HER2/neu tumors.** SCID-beige mice were inoculated s.c. with 1 x 10^6 CT26-HER2/neu tumor cells on day 0. On days 6-10, the mice were treated with daily i.v. injection of PBS or PBS containing 1 µg IL-12 equivalent of mscIL-12.her2.IgG3 or 1 µg of rmIL-12. The tumor size was monitored by caliper measurement every other day and tumor volume calculated.

**Figure 6. Anti-tumor activity of rmIL-12 and mscIL-12.her2.IgG3 in BALB/c mice bearing s.c. CT26 tumors.** BALB/c mice were inoculated s.c. with 1 x 10^6 CT26 tumor cells on day 0. On days 6-10, the mice were treated with daily i.v. injection of PBS or PBS containing 1 µg IL-12 equivalent of mscIL-12.her2.IgG3 or 1 µg of rmIL-12. The tumor size was monitored by caliper measurement every other day and tumor volume calculated.

**Figure 7. Distribution of CD3+ and NK1.1+ cells in the liver of tumor bearing animals.** BALB/c mice bearing CT26-HER2/neu tumors were treated on days 6-10 with PBS, rmIL-12 or mscIL-12.her2.IgG3. On days 5, 7, 10 and 13 mice were euthanized. Lymphocytes isolated from the livers (3 mice per group) were stained for CD3 and NK1.1 and analyzed by flow cytometry. Panel A. flow cytometry profile of lymphocytes from liver stained for CD3 and NK1.1 at day 7. Panel B. Percentage of total lymphocytes from the animals that were CD3+/NK1.1+, CD3+/NK1.1- or CD3-/NK1.1+.
Figure 8. Isotype of anti-HER2/neu antibody response. Plates were coated with HER2/neu ECD\textsuperscript{HER2}. The serum from mice treated with PBS or 1 and 5 μg IL-12 equivalents/day of mscIL-12.her2.IgG3 or rmIL-12 collected at day 20 was pooled, diluted 1:50 and added. Rat anti-mouse IgG1 and anti-mouse IgG2a antibodies were used as primary antibodies. Alkaline phosphatase conjugated goat anti-rat secondary antibody followed by substrate were added and the plates were read at 410 nm.

Figure 9. Anti-angiogenic activity. BALB/c mice bearing s.c. CT26-HER2/neu tumors were treated on days 6-10 with PBS, rmIL-12 or mscIL-12.her2.IgG3. On days 5, 7, 10 and 13 mice (3 mice per group) were euthanized. Tumor sections were stained for PE-CAM (a cell adhesion molecule on endothelial cells) and counterstained with nuclear fast red. Panel A represent tumor sections at day 13 after tumor challenge (day 7 after the initiation of treatment). Bar = 100 μm. Panel B: Photographs of the sections (at least 3 fields per tumor section, average 5) were taken and the photographs were overlaid with a 5x5 μm grid and vessel intersections counted. The number of intersections per view was averaged for all tumors within the same treatment group at the same time point and standard deviations calculated and plotted.
Figure 2
Figure 4
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Figure 9
A recombinant IgG3-(IL-2) fusion protein for the treatment of human HER2/neu expressing tumors

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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 human IgG3 fusion protein containing interleukin-2 (IL-2) fused at its carboxyl terminus. Anti-HER2/neu IgG3-(IL-2) retains antibody and cytokine related activity. Treatment of immunocompetent mice with this antibody fusion protein resulted in significant retardation in the subcutaneous (s.c.) growth of CT26-HER2/neu tumors suggesting that anti-HER2/neu IgG3-(IL-2) fusion protein will be useful in the treatment of HER2/neu expressing tumors. We also found that fusing IL-2 to human IgG3 results in a significant enhancement of the murine anti-human antibody (MAHA) response.

Keywords: Antibodies, Cytokines, Immunotherapy, Cytotoxicity, Antibody Fusion Protein

1. Introduction

The HER2/neu proto-oncogene (also known as c-erbB-2) encodes a 185 kDa transmembrane glycopro-tein 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 [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 antigen (TAA) for tumor specific therapeutic agents. In fact, treatment of patients with advanced breast cancer using the anti-HER2/neu antibody, trastuzumab (Herceptin, Genentech, San Francisco, CA) previously known as rhuMAB 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 anti-tumor 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.

Interleukin-2 (IL-2) is a lymphokine produced by T helper cells which stimulates T cells [17–19] and natural killer (NK) cells [18] and augments antibody dependent cell-mediated cytotoxicity (ADCC) [14,15]. Although it was possible to stimulate an anti-tumor response using high doses of systemically administered
recombinant human IL-2 (rhuIL-2) [21], the systemic administration of high-dose IL-2 had severe toxic side effects [21, 22]. Targeting IL-2 to the site of a tumor with an antibody recognizing a tumor associated antigen is one means of achieving locally high concentrations of IL-2 without toxicity [23–26].

We have now expanded the family of antibody-(IL-2) fusion proteins by developing an anti-HER2/neu IgG3-(IL-2) fusion protein protein that may provide an effective alternative for the therapy of HER2/neu expressing tumors. This novel antibody fusion protein is composed by a human IgG3 with the variable region of the humanized anti-HER2/neu antibody, trastuzumab (Herceptin, Genentech, San Francisco, CA) [13–15] genetically fused to human IL-2. In this report we describe and discuss the strategy of construction and the properties of this novel antibody-(IL-2) fusion protein.

2. Materials and methods

Cell lines: CT26-HER2/neu was developed in our laboratory by transduction of CT26 cells with the cDNA encoding human HER2/neu [14, 15]. It was cultured in Iscove’s Modified Dulbecco’s Medium IMDM (GIBCO, Grand Island, NY) supplemented with 5% bovine calf serum (HyClone, Logan, UT). CTLL-2, an IL-2 dependent murine T cell line (provided by Dr. William Clark, UCLA, CA) was cultured in RPMI 1640 (GIBCO) supplemented with 10% bovine calf serum and IL-2.

Mice: Female BALB/c mice 6–8 weeks of age were obtained from Taconic Farms, Inc. (Germantown, NY).

Vector construction, transfection and initial characterization of anti-human HER2/neu IgG3-(IL-2): For the construction of the heavy chain of human HER2/neu IgG3-(IL-2), the DNA encoding the variable region of trastuzumab [13, 15] was joined to a human γ3 heavy chain containing IL-2 fused at the carboxy terminus of the CH3 domain. It was expressed with the corresponding anti-HER2/neu kappa light chain in P3X63Ag8.653. Stable transfectants were selected and characterized as previously described [13, 15]. The fusion protein was purified from culture supernatants using protein A immobilized on Sepharose 4B fast flow (Sigma Chemical, St. Louis, MO). Purity and integrity were assessed by Coomassie blue staining of proteins separated by SDS-PAGE. The ability of the fusion protein to recognize antigen was assessed by flow cytometry using CT26-HER2/neu cells. The ability of the fusion protein to support the growth of the IL-2 dependent cell line CTLL-2 was determined as previously described [29].

Immunotherapy: 10^6 CT26-HER2/neu cells in 0.15 ml HBSS were injected subcutaneously (s.c.) into the right flank of syngeneic BALB/c mice. Beginning the next day mice randomized into groups of 8 received five daily intravenous (i.v.) injections of 0.25 ml of PBS containing 20 µg of anti-HER2/neu IgG3-(IL-2), the equivalent molar amount of anti-HER2/neu IgG3, or nothing. Tumor growth was monitored and measured with a caliper every three days until day 15 at which time mice were euthanized. Blood samples were collected, serum was separated from clotted blood and stored at −20°C until assayed by ELISA.

Determination of murine anti-human HER2/neu antibodies: Sera from each treatment group were analyzed by ELISA for the presence of antibodies to human IgG3 using 96-well microtiter plates coated with 50 µl of anti-human HER2/neu IgG3 at a concentration of 1 µg/ml. Alkaline phosphatase (AP)-labeled goat anti-mouse IgG (Sigma Chemical, St. Louis, MO) or rat antibodies specific for murine IgG2a, IgG2b, IgG3, IgG1 or kappa (Pharmingen, San Diego, CA) followed by alkaline phosphatase (AP)-labeled goat anti-rat IgG (Pharmingen, San Diego, CA) were used to detect bound murine antibodies. All ELISAs for comparison of titers between the experimental groups were made simultaneously in duplicate using an internal positive control curve for each plate.

Statistical analysis: Statistical analysis of the titration ELISA and anti-tumor experiment was done using a two-tailed Student’s t-test. For all cases results were regarded significant if p values were ≤ 0.05.

3. Results

Anti-HER2/neu IgG3-C1H3-(IL-2) was constructed and expressed as previously described for similar IL-2 fusion proteins [23]. Heavy and light chains of the expected size were synthesized, assembled and secreted. The fusion protein specifically bound to the human HER2/neu expressed on the surface of the murine cell line CT26-HER2/neu and was able to stimulate the proliferation of the IL-2 dependent cell line CTLL-2 with a similar proliferative response observed with equimolar IL-2 concentrations of rhuIL-2 and anti-HER2/neu IgG3-(IL-2) (data not shown).

To investigate in vivo anti-tumor 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 8 received five daily i.v. injections of 0.25 ml of PBS containing 20 μg of anti-HER2/neu IgG3-(IL-2), the equivalent molar amount of anti-HER2/neu IgG3 or nothing. Tumor growth was monitored and measured with a caliper every three days until day 15. The volume was calculated for each mouse of each treatment group, PBS (panel A), anti-HER2/neu IgG3 (panel B), and anti-HER2/neu IgG3-(IL-2) (panel C).

Fig. 1. Anti-Tumor Activity of anti-HER2/neu IgG3-(IL-2) 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 8 mice received five daily i.v. injections of 0.25 ml of PBS containing 20 μg of anti-HER2/neu IgG3-(IL-2), the equivalent molar amount of anti-HER2/neu IgG3 or nothing. Tumor growth was measured with a caliper every three days until day 15. The volume was calculated for each mouse of each treatment group, PBS (panel A), anti-HER2/neu IgG3 (panel B), and anti-HER2/neu IgG3-(IL-2) (panel C).

cally fused to immunostimulatory molecules such as the cytokine IL-12 [30], the costimulatory molecule B7.1 [31], and now IL-2. Targeting IL-2 to the site of a tumor with an antibody-(IL-2) fusion proteins recognizing TAAs has been an effective approach to specifically eliminate many tumors [23,26].

A number of factors were considered in the design of our anti-HER2/neu IgG3-(IL-2) fusion protein. Human IgG3 was chosen because its extended hinge region should provide spacing and flexibility thereby facilitating simultaneous antigen and receptor binding [32,33]. IgG3 is also effective in complement activation [34], and binds FcγRs [34]. IL-2 was used because of its potent immunostimulating properties [17-20,35] and because targeting IL-2 to the site of a tumor with an antibody-(IL-2) fusion protein recognizing TAAs has been an effective approach for specifically eliminating many tumors [23–26]. Antibody-(IL-2) fusion proteins recognizing TAAs have shown superior anti-cancer activity compared with an equivalent amount of free antibody and IL-2 or non-tumor specific antibody cytokine fusion proteins [23,26]. Human IL-2 was used so that the resulting fusion protein was mostly human. Human IL-2 is active in mice making it possible to perform in vivo studies using immune competent mice bearing human HER2/neu expressing tumors.

A single chain Fv specific for peptide epitopes of HER2/neu presented by HLA-A*0201 molecules genetically fused to IL-2 [36] (neo-Ab-IL-2) was found to enhance tumor cell eradication by HER2/neu-specific CD8⁺ T cells in an adoptive transfer model in SCID mice. Surprisingly, the combination of non-tumor-specific CD8⁺ T cells and fusion protein also induced a significant delay of tumor growth, suggesting the potential use of this molecule for redirecting non-tumor-specific T cells to eliminate tumors [36]. However,
in contrast with immunoglobulins such as IgG3, scFvs do not have an Fc. Fc associated functions such as ADCC (an activity that can be enhanced by the presence of IL-2) may play a role in the anti-tumor activity of recombinant antibodies or antibody-(IL-2) fusion proteins. In fact, ADCC has been proposed as a possible mechanism for the clinical response observed with trastuzumab (Herceptin, Genentech, San Francisco, CA) [15]. In addition, studies from other laboratories have shown that while a mouse-human chimeric anti-Id IgG1-mouse IL2 fusion protein (chS5A8-IL2) was effective in the in vivo eradication of the 38C13 tumor, an anti-Id scFv-IL2 fusion protein (scFvS5A8-IL2) containing the variable regions of the chS5A8-IL2 failed to confer protection. These studies suggested that the Fc effector functions such as ADCC contributed to the anti-tumor activity against 38C13 [24]. It is therefore possible that an anti-HER2/neu IgG3-(IL-2) will be superior to anti-HER2/neu scFv-(IL-2) in its anti-tumor activity.

We have found that treatment with anti-HER2/neu IgG3-(IL-2) causes a significant retardation in the growth of s.c. CT26-HER2/neu tumors under conditions in which anti-HER2/neu failed to confer protection. However, we did not observe complete tumor eradication in any mice. Several factors could explain the 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 the tumor model may not be ideal. In addition, we found that treatment with anti-HER2/neu IgG3-(IL-2) results in a dramatic increase in the murine anti-human antibody (MAHA) response. This humoral immune response may be sufficient to neutralize multiple injections of the antibody fusion protein.

It is possible that that an anti-human IgG immune response will not pose a problem in humans, and anti-HER2/neu IgG3-(IL-2) may be even more effective in patients than in mice. However, the IL-2 in the antibody fusion protein may act as an adjuvant to elicit an im-
immune response against the variable regions of humanized or chimeric antibodies. In addition, even though each component of the antibody-(IL-2) fusion protein may not be antigenic by itself in humans, the novel combination of components may produce neoantigenic determinants that will elicit an immune response. Although in certain cases this enhancement of the immune response may be a serious drawback for the therapeutic use of antibody-(IL-2) fusion proteins, in other cases it may be irrelevant [37] or even an advantage [38,39].

In conclusion, our results suggest that an anti-HER2/neu IgG3-(IL-2) fusion protein containing human IL-2 may be an effective therapeutic in patients with tumors overexpressing HER2/neu. The combination of an anti-HER2/neu antibody with IL-2 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 anti-tumor immune response through the immunostimulatory activity of IL-2. In addition, the anti-HER2/neu IgG3-(IL-2) fusion protein may be effective against tumor cells which express a truncated form of ECDHER2 lacking the receptor function rendering them particularly resistant to anti-HER2/neu antibody therapy [14]. Because of IL-2's ability to elicit an immune response to associated antigens (as observed with the dramatically increased immune response against human IgG3), it is also possible that association of anti-HER2/neu IgG3-(IL-2) with soluble ECDHER2 shed by tumor cells will enhance the anti-tumor immune response against ECDHER2. Secretion of ECDHER2 has been reported to be a serious drawback for anti-HER2/neu therapy in humans [14,15].

Finally we would like to stress that anti-HER2/neu IgG3-(IL-2) would not be a replacement for trastuzumab (Herceptin, Genentech, San Francisco, CA), but instead would provide an alternative therapy to be used in combination with the antibody or other anti-cancer approaches. These approaches might include chemotherapy or other anti-HER2/neu antibody fusion proteins such as anti-HER2/neu with the costimulator B7.1 [31] or the cytokine IL-12 [30]. The availability of more than one antibody fusion protein will allow us to explore potentials synergistic effects that may be obtained from manipulating the immune response.

Acknowledgments

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-CA09120-25 from NCI (NIH), Cancer Center Core grant CA-16042 (UCLA) and grant HMP-98-B-1-0001 from the Korean Ministry of Health and Welfare. We are grateful to Dr. Paul Carter for the DNA encoding the variable domains of the humanized antibody hum 4D5.8, and to Dr. Donald Morrison for assistance with statistical analysis.

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