Cytokines fused to antibodies and their combinations as therapeutic agents against different peritoneal HER2/*neu* expressing tumors

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Abstract

We have previously generated antihuman HER2/*neu*–humanized IgG3 fused to interleukin-2 (IL-2), IL-12, or granulocyte macrophage colony-stimulating factor (GM-CSF) [monofunctional fusion proteins (mono-AbFP)] or fused to IL-2 and IL-12 or IL-12 and GM-CSF [bifunctional fusion proteins (bi-AbFP)]. These AbFPs retained cytokine and antigen-binding activities. We have now further characterized the AbFPs and determined the heparin-binding activity of the fused cytokines, their ability to trigger IFN-γ secretion and natural killer (NK) activation, and their direct antitumor efficacy. Flow cytometry revealed heparin-binding activity in the AbFPs containing IL-12 and IL-2, although this activity seems to be decreased in the bi-AbFPs. However, both bi-AbFPs retained the capacity to stimulate IL-12-dependent IFN-γ secretion in the NK cell line KY-1, and IL-12/IL-2 bi-AbFP induced NK activity in splenocytes. The antitumor effectiveness of bi-AbFPs and mono-AbFP combinations was studied in mice challenged i.p. with three different human HER2/*neu* murine syngeneic models (D2F2/E2, CT26-HER2/*neu*, and MC38-HER2/*neu*). Although a significant variability in the profile of antitumor response was observed in the different tumor models, the combination of IL-12 and GM-CSF mono-AbFPs protected 100% of D2F2/E2-challenged and 75% of CT26-HER2/*neu*–challenged mice. In contrast, bi-AbFPs protected less than the combination of mono-AbFPs and, in some models, even less than mono-AbFPs alone. However, in all cases, most of long-term survivors showed protection after s.c. rechallenge with the tumors and later with the parental tumors not expressing HER2/*neu*. These results show that, although the pattern of protection is tumor model dependent, treatments with AbFPs can effectively generate high levels of protection against peritoneal tumors expressing HER2/*neu*, which may be relevant in patients with primary or metastatic peritoneal carcinomatosis that may be observed in ovarian, colon, stomach, bladder, lung, and breast cancers. [Mol Cancer Ther 2006;5(4):1029–40]

Introduction

The HER2/*neu* proto-oncogene, also known as *erbB-2*, encodes a 185-kDa type I transmembrane receptor tyrosine kinase that is member of the epidermal growth factor receptor family (1–3). Early studies have identified HER2/*neu* protein overexpression in several human carcinomas, including subsets of ovarian and breast cancers (4–6). HER2/*neu* overexpression has also been linked to a short relapse time and poor survival of breast cancer patients (7), suggesting that it may play an important role in the molecular mechanisms of human cancers. These characteristics (extracellular accessibility, high expression, and association with poor prognosis) make HER2/*neu* an attractive candidate for antibody therapy. Metastatic breast cancer patients are currently being treated with Trastuzumab (also known as Herceptin; Genentech, Inc., San Francisco, CA), a Food and Drug Administration–approved humanized monoclonal anti-HER2/*neu* (8). Breast cancer clinical trials for patients with advanced disease expressing high levels of HER2/*neu* showed that the use of Trastuzumab as a single immunotherapeutic agent resulted in a objective response rate of 12% to 26% (9–11). Subsequent clinical trials in patients with advanced disease have also shown that targeting metastatic breast cancer with Trastuzumab combined with chemotherapy resulted in a 50% objective response, but disease relapse still affected most cases (12). In addition, Trastuzumab lacks considerable activity against tumors expressing HER2/*neu* that are not from breast origin (13). Furthermore, resistance to Trastuzumab is becoming a problem in patients with breast tumors (13), suggesting that novel treatment for patients with HER2/*neu*–expressing tumors are still needed.

The cytokines interleukin-2 (IL-2), IL-12, and granulocyte macrophage colony-stimulating factor (GM-CSF) are known to be potent immunostimulators. Several cytokines, such as IL-2 and IL-12, are known to bind not only to their membrane receptors but also to glycosaminoglycans particularly the heparin/heparan sulfate family (14, 15). The

Received 11/28/05; revised 12/24/05; accepted 1/25/06.

Grant support: National Cancer Institute/NIH grants CA86915 and CA107023 and the 2002 AACR-California Department of Health Services Career Development Award in Gender-Related Cancer Research.

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doi:10.1158/1535-7163.MCT-05-0488

binding to glycosaminoglycans may have a number of functions, among them, to favor paracrine distribution from their secretion source and modulate their circulating half-life (16). Specifically, in the case of IL-2, it is important in the propagation and control of the T-cell response (17), whereas for IL-12, it is suggested to play a major role in the regulation of IFN-γ secretion (18). IL-2, IL-12, and GM-CSF have been shown to activate potent immune response against non-immunogenic tumors (19–21). Moreover, the combined use of IL-2 and IL-12 or GM-CSF and IL-12 was more effective than single cytokines in cancer treatment (22–25). However, the systemic administration of cytokines necessary to achieve an effective dose in the tumor area has often been associated with severe toxicity (26–28). Antibody fusion proteins (AbFP) targeting tumor-associated antigens have been used in preclinical studies as a strategy to concentrate cytokines in the tumor microenvironment as direct therapeutics to selectively target the tumor and simultaneously enhance the tumoricidal activity of the antibody and the immune response against the tumor (29, 30). In addition, AbFPs complexed to the tumor antigen can be used as adjuvants in prophylactic cancer vaccination to enhance the immune response against the tumor cells (31, 32). Recently, an AbFP anti-GD2 IgG1 fused to IL-2 (immunocytokine EMD 273063) has been tested in phase I clinical trials as a direct therapeutic in patients with metastatic melanoma, resulting in immune activation with acceptable toxicity in patients, and is now in plan for phase II testing (33).

To improve the efficacy of treatment against cancers expressing HER2/*neu*, we fused different cytokines to an antihuman HER2/*neu* consisting of the variable region of the humanized Trastuzumab antibody and the constant region of human IgG3. Human IgG3 has an extended hinge region, which provides spacing and flexibility (34–36), therefore facilitating the simultaneous binding by the AbFPs to the antigen and the different cytokine receptors. IgG3 is also the most effective human IgG isotype for complement activation and binds both human and murine FcγRs (37, 38). The antihuman HER2/*neu* IgG3 was genetically fused to human IL-2 [IgG3-(IL-2); ref. 39], murine single chain IL-12 [(IL-12)-IgG3; ref. 40], or murine GM-CSF [IgG3-(GM-CSF); ref. 41]. Recently, we also developed two novel anti-HER2/*neu* AbFPs with IL-12 genetically fused to the NH_2 terminus and human IL-2 [(IL-12)-IgG3-(IL-2)] or murine GM-CSF [(IL-12)-IgG3-(GM-CSF)] fused to the COOH terminus (bi-AbFPs; ref. 32). We formerly showed that all AbFPs are properly assembled, retain cytokine activity, and specifically target the extracellular domain of human HER2/*neu*, and that AbFPs alone or combined can be effective adjuvants of the extracellular domain of human HER2/*neu* in a prophylactic vaccination setting against rat HER2/*neu*–expressing tumors (31, 32). The antibody fusion proteins containing a single cytokine (mono-AbFPs) have been effective as direct antitumor agents inhibiting the growth of murine tumors expressing human HER2/*neu* (39–41). However, the bi-AbFPs or the combination of mono-AbFPs have not been tested *in vivo* as direct immunotherapeutics. In the present work, we further characterize the *in vitro* properties of the AbFPs. We studied their ability to bind to HER2/*neu* expressed on the surface of cancer cells as well as the ability of the fused cytokines to bind glycosaminoglycans present on the surface of cells (heparin-binding activity). We also determine the capacity of mono-AbFPs and bi-AbFPs to stimulate IL-12-dependent IFN-γ secretion by the natural killer (NK) cell line KY-1, an activity that has been associated with the heparin-binding activity of IL-12 (18). In addition, we tested *in vitro* the AbFPs capacity to induce NK activity. Finally, we investigated the *in vivo* efficacy of the AbFPs and combinations of mono-AbFPs as direct therapeutics using different human HER2/*neu*–expressing murine models of peritoneal tumors.

Materials and Methods

Animals

Female BALB/c and C57BL/6 mice 6 to 8 weeks old were obtained from Taconic Farms (Germantown, NY). Mice were housed under specific pathogen-free conditions. The facility was maintained at 20°C and 55% relative humidity with 10 to 15 air exchanges per hour. The experiments were done following guidelines of the NIH to minimize the discomfort of the animals.

Cell Lines and Culture Conditions

The murine myeloma cell line P3X63Ag8.653 (American Type Culture Collection, Manassas, VA) and its derivatives expressing AbFPs, the murine colon carcinomas of epithelial origin [CT26 (syngeneic for BALB/c); generously provided by Dr. Young Chul Sung, University of Science and Technology, Pohang, South Korea and MC38 (syngeneic for C57BL/6); generously provide by Dr. Jeffrey Schlom, National Cancer Institute, Bethesda, MD], the derivatives transduced with the cDNA-encoding human HER2/*neu* (CT26-HER2/*neu* and MC38-HER2/*neu*; ref. 42), and the NK-susceptible lymphoma cell line yeast artificial chromosome-1 (YAC-1; American Type Culture Collection) were grown in Iscove's modified Dulbecco's medium (Irvine Scientific, Irvine, CA) supplemented with 2 mmol/L L-glutamine, 10 units/mL penicillin, and 10 μg/mL streptomycin (GPS; Sigma Chemical, St. Louis, MO) and 5% calf serum (Atlanta Biologicals, Norcross, GA). The mouse mammary tumor cell lines of epithelial origin D2F2 syngeneic for BALB/c mice and D2F2/E2 transfected with a vector encoding human HER2/*neu* (43) were kindly provided by Dr. Wei-Zen Wei (Wayne State University, Detroit, MI). D2F2 and D2F2/E2 cells were cultured in Iscove's modified Dulbecco's medium supplemented with GPS and 10% calf serum. The murine NK cell line KY-1 (generously given by Prof. Wayne M. Yokohama, M.D., Washington University, St. Louis, MO) was grown in RPMI 1640 supplemented with 10% calf serum, GPS, 5 mmol/L 2-mercaptoethanol, and 100 units/mL recombinant human IL-2 (PeproTech, Rocky Hill, NJ).

Generation and Purification of AbFPs

The construction, purification, and characterization of the anti-HER2/*neu* IgG3, IgG3-(GM-CSF), IgG3-(IL-2),

(IL-12)-IgG3, (IL-12)-IgG3-(GM-CSF), and (IL-12)-IgG3-(IL-2) cytokine fusion proteins have been described previously (32, 39–41). Briefly, IgG3 and the AbFPs were constructed by joining the DNA sequence of human IgG3 heavy and light chain constant regions with the variable region of the anti-HER2/*neu* Herceptin. For the construction of the AbFPs, we used human IL-2, murine IL-12, and murine GM-CSF, all of which are active in mouse (28, 39, 44). We used human IL-2 and murine IL-12 and GM-CSF because human IL-2 exhibits full bioactivity in mice, whereas human IL-12 and GM-CSF are not functional in mice. The cytokines were genetically fused to the extremes of the heavy chain: IL-12 to the NH_2 terminus and IL-2 and GM-CSF to the COOH terminus (Fig. 1). Constructs were transfected in myeloma cell lines, and the secreted antibodies were purified by affinity chromatography with Protein A columns (Sigma Chemical). The integrity and quantification of the purified protein was confirmed by SDS-PAGE.

Antibody Binding to HER2/neu Expressed in Tumor Cells

CT26 or CT26-HER2/*neu* cells (10^6) were incubated in the presence of 2 μg anti-HER2/*neu* IgG3, or the equivalent molar amount of (IL-12)-IgG3-(GM-CSF), (IL-12)-IgG3-(IL-2), IgG3-(GM-CSF), (IL-12)-IgG3, IgG3-(IL-2), or anti-dansyl IgG3 (isotype-matched control) for 2 hours at 4°C. The cells were washed and then incubated 30 minutes at 4°C with biotin-conjugated goat anti-human IgG (PharMingen, San Diego, CA), washed again, incubated 30 minutes at 4°C with streptavidin-phycoerythrin (PharMingen), and analyzed by flow cytometry. Analysis was done with a FACScan (Becton Dickinson, Mountain View, CA) equipped with a blue laser excitation of 15 mW at 488 nm. Alternatively, we used as secondary antibody a FITC-conjugated anti-human monoclonal antibody specific for human κ chain (PharMingen). To block AbFPs cell surface binding through the cytokine heparin-binding site, 5,000 units/mL heparin from porcine intestinal mucosa (Sigma, St. Louis, MO) was added as competitor and incubated with anti-HER2/*neu* IgG3, (IL-12)-IgG3, IgG3-(IL-2), (IL-12)-IgG3-(IL-2), or (IL-12)-IgG3-(GM-CSF) in PBS overnight at 4°C. The next day, CT26 and CT26-HER2/*neu* cells were incubated with the mixture of heparin and AbFPs for 4 hours at 4°C and binding detected by flow cytometry as described above.

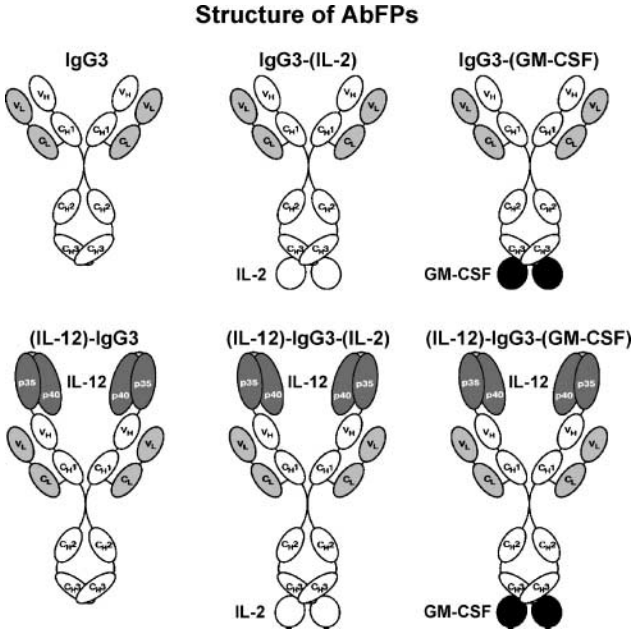

Figure 1. Schematic representation of anti-HER2/*neu* IgG3 and the cytokine fusion proteins IgG3-(IL-2), IgG3-(GM-CSF), (IL-12)-IgG3, (IL-12)-IgG3-(IL-2), and (IL-12)-IgG3-(GM-CSF). The constructs including IL-2 and GM-CSF have the cytokine sequences fused directly to the COOH terminus of the constant region of the IgG3 sequence, whereas single-chain IL-12 p40 and p35 domains are joined together and to the variable regions (NH_2 terminus) of the IgG3 heavy chain sequence through flexible $(Gly_4Ser)_3$ linkers.

Assay for IL-12-Dependent IFN-γ Secretion

For IFN-γ secretion studies, KY-1 cells were washed and incubated for 18 hours in 96-well plates at 37°C in 5% CO_2 in the presence of serial 1:5 dilutions ranging from 10 to 0.08 ng/mL of equivalent molar concentrations of IgG3-(IL-2), (IL-12)-IgG3, IgG3-(GM-CSF), a mixture of IgG3-(IL-2) and (IL-12)-IgG3, a mixture of (IL-12)-IgG3 and IgG3-(GM-CSF), (IL-12)-IgG3-(IL-2), or (IL-12)-IgG3-(GM-CSF). After incubation, supernatant aliquots diluted 1:20 were added into 96-well plates precoated with anti-mouse IFN-γ capture antibody (PharMingen) and incubated overnight at 4°C. The following day the plates were washed, a detecting anti-mouse IFN-γ biotin-conjugated antibody (PharMingen) added, and the plates were incubated at 37°C for 1 hour. The plates were then washed and incubated in the presence of streptavidin-alkaline phosphatase (Sigma) at 37°C for 1 hour. After washing, *p*-nitrophenyl phosphate disodium dissolved in diethanolamine buffer was added, and the plates were incubated at room temperature for 45 minutes and read at A_{410}. Quantitation of results was done using a murine IFN-γ (PharMingen) standard curve generated in each plate. All measurements were made in quadruplicate.

Mouse Splenocyte Isolation, Stimulation with AbFPs, and Determination of NK Cytotoxicity

Splenocyte Isolation and Stimulation. Spleens from naive 6-week-old female BALB/c mice (Taconic Farms) were removed, and a single cell preparation was made by gently teasing the spleens against sterile glass slides and passing the cells through a 100-μm cell strainer (Becton Dickinson Labware, Franklin Lanes, NJ) to remove large debris. RBC were lysed using 0.85% ammonium chloride, and the splenocytes were washed twice in RPMI 1640 supplemented with 10% calf serum, GPS, and 5 mmol/L 2-mercaptoethanol (31). A total of 3×10^6 cells were resuspended in 3 mL RPMI 1640 supplemented with 10% calf serum, GPS, and 5 mmol/L 2-mercaptoethanol and containing 7.14 nmol/L IgG3-(IL-2), (IL-12)-IgG3, IgG3-(GM-CSF), a mixture of IgG3-(IL-2) and (IL-12)-IgG3, a mixture of (IL-12)-IgG3 and IgG3-(GM-CSF), (IL-12)-IgG3-(IL-2), or (IL-12)-IgG3-(GM-CSF) and incubated for 18 hours at 37°C in 5% CO_2.

NK Cytotoxicity Assay. NK activity was measured by a standard ^{51}Cr release assay using NK-sensitive YAC-1 cells.

Briefly, 10^6 YAC-1 tumor cell targets were incubated with 1 mCi/mL ^{51}Cr (New England Nuclear, Boston, MA) in RPMI 1640 supplemented with 20% calf serum for 4 hours at 37°C. During this incubation time, activated splenocytes were washed, counted, and loaded into 96-well round-bottom plates (Costar Corning, Cambridge, MA) at 5×10^5 cells per well in RPMI 1640 supplemented with 10% calf serum, GPS, and 5 mmol/L 2-mercaptoethanol. Once the effector cells were plated, the target cells were washed twice with media and seeded over the effector cells at 10^4 per well with effector/target cell ratios from 50:1 to 1:1 in 200 μL/well. Plates were centrifuged for 4 minutes at $1{,}000 \times g$. Following incubation with the effector cells for 4 hours, plates were centrifuged for 4 minutes at $1{,}000 \times g$; 100 μL supernatant from each well were harvested and transferred into counting vials. Radioactivity was measured in a Gamma 5000 counter (Beckman Coulter, Fullerton, CA), and specific lysis was determined according to the following formula:

$$\text{specific lysis } (\%) = 100 \times (\text{cpm}_{\text{test control}})/(\text{cpm}_{\text{total control}}).$$

To determine $\text{cpm}_{\text{control}}$, target cells were cultured in the absence of effector cells. The $\text{cpm}_{\text{total}}$ was measured after complete lysis of target cells in 1% Triton X-100 (Sigma). The cpm_{test} was determined for all wells with effector and target cells. All assays were done in quadruplicate. Significance in the difference between treatments was determined by two tailed, unpaired t test.

Determination of AbFPs *In vivo* Antitumor Protection

Groups of eight female mice 6 to 8 weeks old were challenged i.p. at day 0 with either 2×10^5 D2F2/E2 cells, 10^6 CT26-HER2/*neu* (in BALB/c mice), or 10^6 MC36-HER2/*neu* (in C57BL/6) in 150 μL HBSS (Life Technologies, Rockville, MD). Minimum tumorigenic doses were previously established and defined as the minimum number of cells injected i.p. required to achieve 100% tumor progression. Thereafter, animals were treated with one daily i.p. injection for 3 days of PBS, 2.3 μg of (IL-12)-IgG3, or the equivalent amount of (IL-12)-IgG3-(IL-2), or (IL-12)-IgG3-(GM-CSF). A dose of 2.3 μg represents the equivalent of 1 μg of IL-12, previously shown to effectively elicit tumor protection in s.c. CT26-HER2/*neu* tumor models (40). A second experiment excluded the MC38-HER2/*neu* model but included the previous treatments plus IgG3, IgG3-(IL-2), IgG3-(GM-CSF), (IL-12)-IgG3, a mixture of (IL-12)-IgG3 and IgG3-(IL-2), and a mixture of (IL-12)-IgG3 and IgG3-(GM-CSF). Differences in survival between experimental groups were determined by the nonparametric Peto-Peto-Wilcoxon test. Long-term survivors and a cohort of age-matched control mice were rechallenged s.c. on the left flank with a the same dose of CT26-HER2/*neu* and D2F2/E2 and monitored for 40 days. Tumor progressions were measured with caliper every other day, and once the tumor reached 15-mm diameter, the mice were euthanized. Rechallenged survivors and a new cohort of age-matched control mice were subsequently challenged s.c. contralaterally on the right flank with the parental cell lines CT26 (10^6 cells) and D2F2 (2×10^5 cells) and monitored every other day for tumor growth. Once the tumor reached 15-mm diameter, the mice were euthanized.

Results

Antigen and Heparin-Binding Activity

The construction, expression, and characterization of the anti-HER2/*neu* AbFPs (IL-12)-IgG3, IgG3-(IL-2), IgG3-(GM-CSF), (IL-12)-IgG3-(IL-2), and (IL-12)-IgG3-(GM-CSF) (Fig. 1) have been described previously (32, 39–41). Bi-AbFPs were previously shown to retain cytokine activity and bind the extracellular domain of human HER2/*neu* by ELISA (32). To further characterize the *in vitro* properties of the AbFPs, we tested the ability of the bi-AbFP to target the HER2/*neu* antigen expressed on the surface of tumor cells by flow cytometry because the specific binding to tumor cells is required in the context of a therapeutic setting. The murine colon carcinoma cell line CT26-HER2/*neu* and the CT26 parental cell line not expressing the antigen were used as positive and negative controls, respectively. Figure 2 shows that anti-HER2/*neu* IgG3, the mono-AbFPs, and the bi-AbFPs, but not anti-dansyl IgG3, bind to CT26-HER2/*neu*. There was also less intense but still significant binding to the parental cell line CT26 by (IL-12)-IgG3 and to a lesser extent by IgG3-(IL-2), (IL-12)-IgG3-(IL-2), and (IL-12)-IgG3-(GM-CSF) (Fig. 2). This observation is consistent with the presence of a heparin-binding site on IL-12, which results in binding to glycosaminoglycans present on the cell surface with an estimated binding constant of 10 nmol/L (15). IL-2 also binds to heparin and other glycosaminoglycans but with an estimated binding constant of only 0.5 μmol/L (45). We did not detect any binding of IgG3-(GM-CSF) to CT26 cells in spite of the fact that GM-CSF binding to glycosaminoglycans has been previously described (46, 47).

Surprisingly, (IL-12)-IgG3-(IL-2) binding to CT26 was less than that observed using (IL-12)-IgG3, although both IL-12 and IL-2 contain heparin-binding sites and were present in the same molecule (Fig. 2). In addition, (IL-12)-IgG3-(GM-CSF) showed a dramatic reduction in binding to CT26 compared with (IL-12)-IgG3. Similar results were obtained using a FITC-conjugated anti-human κ monoclonal antibody to detect the AbFPs binding to the cell surface (data not shown). Thus, the combination of IL-12 with either of the other two cytokines on the same IgG3 molecule seems to diminish the heparin-binding activity of IL-12.

To confirm that the binding of the AbFPs to CT26 was through the heparin-binding site of IL-12 and IL-2, all AbFPs were preincubated with heparin and then incubated with CT26 or CT26-HER2/*neu*. Binding of (IL-12)-IgG3, IgG3-(IL-2), and both bi-AbFPs to CT26 but not to CT26-HER2/*neu* was completely abolished by the incubation in the presence of heparin (Fig. 3). Thus, the bi-AbFPs retain the ability to bind the antigen expressed on the surface of tumor cells, and the fusion of the two cytokines to both ends of the heavy chain of IgG3 does not compromise its

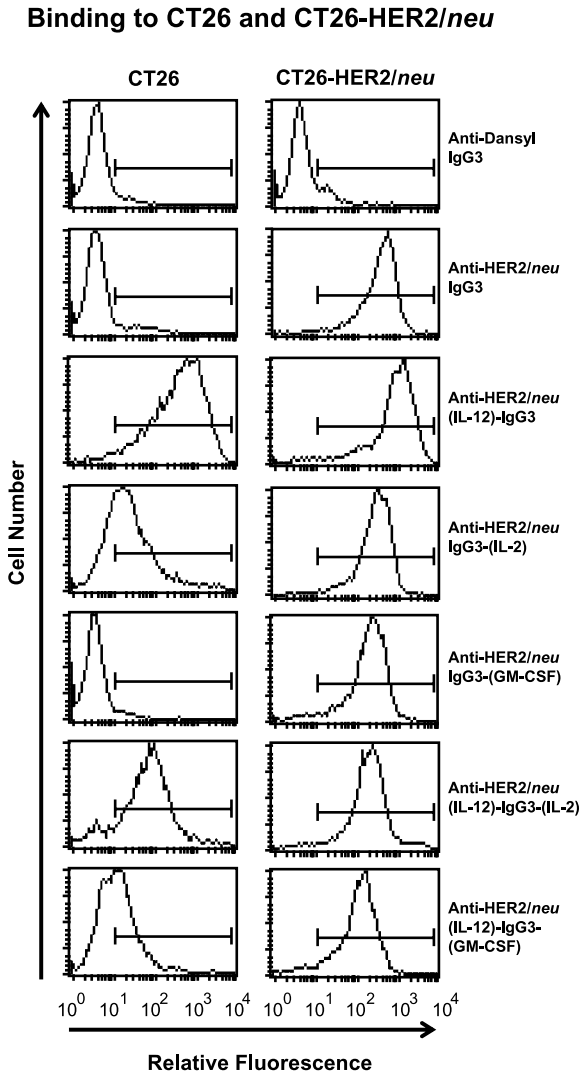

Figure 2. Binding of anti-HER2/*neu* AbFPs to CT26 and CT26-HER2/*neu* as determined by cytometry. CT26 and CT26-HER2/*neu* cells were incubated with anti-dansyl IgG3 (isotype negative control), anti-HER2/*neu* IgG3, IgG3-(IL-2), IgG3-(GM-CSF), (IL-12)-IgG3, (IL-12)-IgG3-(IL-2), or (IL-12)-IgG3-(GM-CSF) followed by biotin-conjugated goat antihuman IgG and streptavidin-phycoerythrin. Note the binding of IgG3-(IL-2), (IL-12)-IgG3, (IL-12)-IgG3-(IL-2), and (IL-12)-IgG3-(GM-CSF) to the HER2/*neu* – non-expressing cell line CT26.

ability to bind HER2/*neu*. However, it should be noted that all antibodies with mscIL-12 fused to the NH_2 terminus of V_H show decreased binding to HER2/*neu* present on the surface of cells in the presence of heparin (Fig. 3).

IL-12-Dependent IFN-γ Secretion

We used the murine NK cell line KY-1 to determine the ability of the bi-AbFP to stimulate IL-12-dependent IFN-γ secretion. This cell line, which expresses the IL-12R, has been used to show that the binding of IL-12 to surface glycosaminoglycans through its heparin-binding site is important for the induction of IFN-γ secretion (18). Figure 4 shows that both anti-HER2/*neu* bi-AbFPs retained the capacity to stimulate IFN-γ secretion, whereas neither IgG3-(IL-2) nor IgG3-(GM-CSF) alone stimulated the secretion of IFN-γ (Fig. 4A and B). Although (IL-12)-IgG3-(IL-2) showed slightly reduced IFN-γ secretion compared with the IL-12 controls, this effect can not be explained by the apparent reduction in its heparin-binding activity. The (IL-12)-IgG3-(GM-CSF) bi-AbFP, which exhibits even less heparin-binding activity, also shows the same IFN-γ secretion profile as IL-12 alone. Therefore, the apparent impairment in IL-12 heparin binding exhibited by the bi-AbFPs did not affect their induction of IFN-γ secretion by KY-1 cells.

NK Cytotoxicity

The NK activity of splenocytes treated with AbFPs was measured by ^{51}Cr release from NK-sensitive YAC-1 cells (Fig. 5). The combined use of (IL-12)-IgG3 and IgG3-(IL-2) resulted in significantly higher cytotoxicity compared with all the other treatments, whereas (IL-12)-IgG3-(IL-2) did not result in increased generation of NK cytotoxicity compared with IgG3-(IL-2) (*t* test, $P > 0.06$). Consistent with earlier results, the cytotoxicity elicited by IgG3-(IL-2) was significantly higher than that elicited by free IL-2 alone

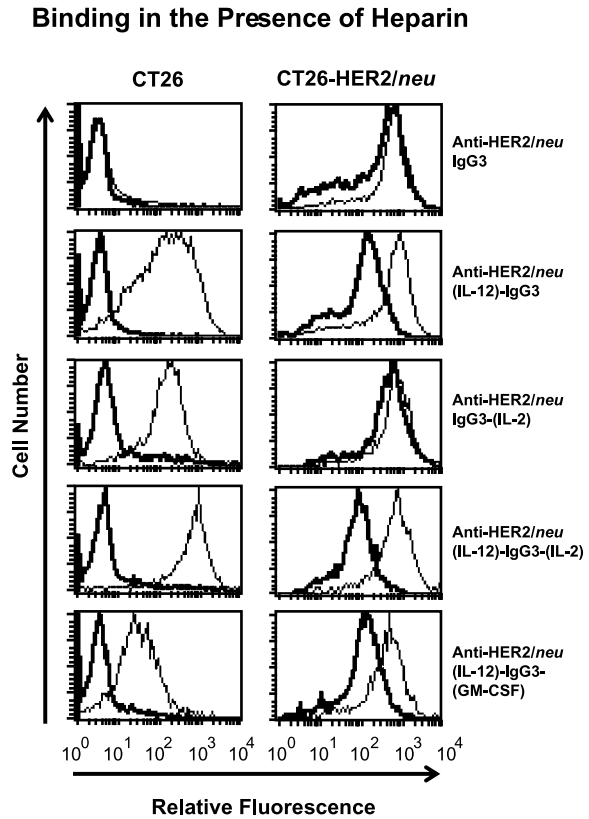

Figure 3. Heparin completely inhibits the binding of anti-HER2/*neu* (IL-12)-IgG3, IgG3-(IL-2), (IL-12)-IgG3-(IL-2), and (IL-12)-IgG3-(GM-CSF) to CT26 but not to CT26-HER2/*neu*. CT26 and CT26-HER2/*neu* were incubated with anti-HER2/*neu* IgG3, or equivalent molar amount of (IL-12)-IgG3, IgG3-(IL-2), (IL-12)-IgG3-(IL-2), or (IL-12)-IgG3-(GM-CSF) in the presence (*thick line*) or absence (*light line*) of heparin followed by biotin-conjugated goat antihuman IgG and phycoerythrin-streptavidin and analyzed by flow cytometry.

(data not shown; ref. 48). Similar experiments using GM-CSF fusion proteins showed that this cytokine did not make a significant contribution to the generation of NK cells in this assay (data not shown).

In vivo Protection Elicited by AbFPs on Different Peritoneal Murine Tumor Models Expressing Human HER2/*neu*

Having shown that bi-AbFPs target HER2/*neu* expressed on the surface of the tumor cells and induce IFN-γ secretion and NK activation, we investigated the *in vivo* activity of bi-AbFPs as direct antitumor agents. As a model of i.p. epithelial tumors, mice were injected i.p. at day 0 with the epithelial cancer cell lines D2F2/E2, CT26-HER2/*neu* syngeneic to BALB/c, or MC38-HER2/*neu* syngeneic to C57BL/6. We evaluated the efficacy of bi-AbFPs in a therapeutic setting compared with that of (IL-12)-IgG3 and PBS when administered i.p. 1, 2, and 3 days after tumor challenge. For initial proof-of-principle, we compared the efficacy of each bi-AbFP to that of (IL-12)-IgG3 because this mono-AbFP has proved to be more effective than IgG3-(IL-2) or IgG3-(GM-CSF) in previous therapeutic settings.

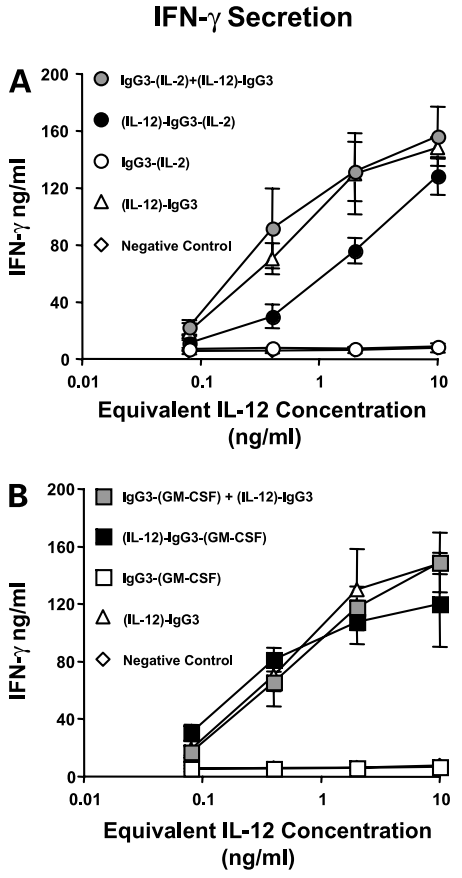

Figure 4. *In vitro* induction of IL-12-dependent secretion of IFN-γ by KY-1 cells. KY-1 cells were incubated for 18 h with serial dilutions of equivalent molar amount of mono-AbFPs either alone or in combination or with the bi-AbFPs. Secreted IFN-γ was detected by ELISA. **A** and **B,** data for fusion proteins containing IL-2 (**A**) and GM-CSF (**B**). Experiments were done in quadruplicate. *Points,* mean; *bars,* SD.

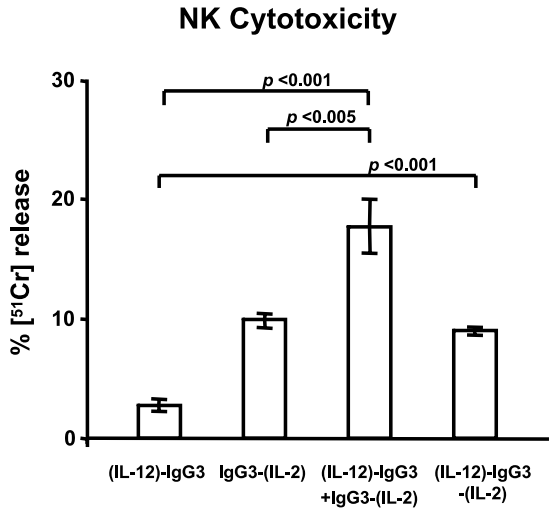

Figure 5. NK cytotoxicity elicited by AbFPs. Splenocyte effector cells were incubated 18 h with 7.14 nmol/L IL-12, IL-2, IL-2 + IL-12, (IL-12)-IgG3, IgG3-(IL-2), IgG3-(IL-2) + (IL-12)-IgG3, or (IL-12)-IgG3-(IL-2). The cytotoxicity was determined by incubating activated splenocyte effector cells with 10^4 ^{51}Cr loaded NK-sensitive YAC-1 target cells at an effector/target ratio of 50:1 for 4 h. *Columns,* mean of quadruplicate determinations; *bars,* SD. *Top,* significance of different comparisons.

As shown in Table 1 and Supplementary Material I[4], PBS control treatment resulted in no protection for all subjects. In contrast, treatment with the bi-AbFPs resulted in a different pattern of protection for each tumor model, whereas (IL-12)-IgG3 resulted in significant protection in all models (≥50% long-term survivors). Both bi-AbFPs elicited protection similar to that of (IL-12)-IgG3 against D2F2/E2 (Supplementary Material IA;[4] Table 1), showed poor protection against CT26-HER2/*neu* challenge when compared with (IL-12)-IgG3 (Supplementary Material IB; Table 1), and showed intermediate protection against MC38-HER2/*neu* (Supplementary Material IC;[4] Table 1). It is important to note that although treatment with bi-AbFPs elicited lower protection against the CT26-HER2/*neu* model than did (IL-12)-IgG3 treatment, the mean survival was significantly higher than for the PBS control ($P < 0.001$; Table 1). These results suggest that treatment with combinations of cytokines fused in one bi-AbFPs can result in effective, poor, or even no protection in different tumor models independent of the mouse strain used.

Because D2F2/E2 exhibited the highest and CT26-HER2/*neu* the lowest protection elicited by the bi-AbFPs, we used these models to examine the efficacy of the combination of mono-AbFPs compared with the bi-AbFPs to address whether the differential protection is attributable to their simultaneous presence in the same AbFP. We compared side by side the protection elicited in the absence of antibodies, in the presence of IgG3 alone, the mono-AbFPs and their combinations, and the bi-AbFPs. As expected, tumors grew rapidly in the control groups treated with PBS

[4] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Table 1. *In vivo* experiment 1: survival of mice challenged with D2F2/E2, CT26-HER2/*neu*, and MC38-HER2/*neu* over a period of 200 days

Tumor model	Treatment	Median survival (d)	Long-term survivors (200 d)	*P**		
				(IL-12)-IgG3 vs	(IL-12)-IgG3-(IL-2) vs	(IL-12)-IgG3-(GM-CSF) vs
D2F2/E2	PBS	35	0/8	<0.005↓	<0.001↓	<0.001↓
	(IL-12)-IgG3	200	5/8	NA †	0.897	0.895
	(IL-12)-IgG3-(IL-2)	136	4/8	0.897	NA	0.913
	(IL-12)-IgG3-(GM-CSF)	157	4/8	0.895	0.913	NA
CT26-HER2/*neu*	PBS	18	0/8	<0.001↓	<0.001↓	<0.001↓
	(IL-12)-IgG3	192	4/8	NA	<0.003↓	<0.001↓
	(IL-12)-IgG3-(IL-2)	26	1/8	<0.003↑	NA	0.432
	(IL-12)-IgG3-(GM-CSF)	26	0/8	<0.001↑	0.432	NA
MC38-HER2/*neu*	PBS	39	0/8	<0.001↓	0.161	0.003↓
	(IL-12)-IgG3	200	5/8	NA	0.065↓	0.215
	(IL-12)-IgG3-(IL-2)	43	3/8	0.065↑	NA	0.121
	(IL-12)-IgG3-(GM-CSF)	56	2/8	0.215	0.121	NA

Abbreviation: NA, not applicable.
*The significance in survival differences between the treatments was determined by Peto-Peto-Wilcoxon test. Arrows indicate significantly higher or lower survival at $P < 0.07$.
†Indicate comparison between the same groups.

and IgG3, with virtually no survivors after 40 days in both tumor models (Supplementary Material II).[4] In the case of D2F2/E2, only treatment with IgG3-(IL-2) did not achieve significant protection compared with the controls under these conditions. All the other treatments resulted in significant protection (Supplementary Material IIA;[4] Table 2). The highest level of protection was observed in the groups injected with the combinations of mono-AbFPs followed by (IL-12)-IgG3, the bi-AbFPs, and IgG3-(GM-CSF). Challenge with CT26-HER2/*neu* showed highest protection by the combination of (IL-12)-IgG3 and IgG3-(GM-CSF) followed by (IL-12)-IgG3, the combination of (IL-12)-IgG3 and IgG3-(IL-2), and marginal but still significant protection by both bi-AbFPs. Note that in this case, IgG3-(IL-2) and IgG3-(GM-CSF) did not show a protection significantly better than PBS or IgG3. These results show that treatment of tumors with a combination of cytokines fused to same antibody as bi-AbFPs does not always result in better protection when compared with a combination of two mono-AbFPs and, in some cases, may even be detrimental. Under these conditions, the overall protection provided by the combination of AbFPs seems superior to that elicited by the bi-AbFPs and most mono-AbFPs alone with the exception of (IL-12)-IgG3, which conferred a superior level of protection in all tumor models compared with the PBS controls.

To determine whether treatment with the AbFPs could elicit immunologic memory and systemic immunity, both long-term survivors and control naive mice were rechallenged s.c. on the right flank with the original dose of the tumor cells (Supplementary Material II).[4] No tumor growth was observed in nearly all long-term survivors, independently of the treatment or tumor model used, whereas tumors grew in all control mice. These results show that the local treatment of intra-abdominal HER2/*neu* expressing tumors with our AbFPs can generate not only an effective protection in mice but also generate an effective systemic immunity that prevent tumor growth after rechallenge. Forty days later, D2F2/E2 and CT26-HER2/*neu* long-term survivors were challenged s.c. on the opposite flank with the parental cell lines that do not express human HER2/*neu* D2F2 and CT26, respectively. All but one of the mice showed tumor protection against the HER2/*neu*–negative parental cell lines 95 days after the challenge, whereas all control mice died between the second and the third week after challenge (data not shown).

Discussion

We have previously developed a family of recombinant antibodies targeting human HER2/*neu* fused to the cytokines IL-12, IL-2, and GM-CSF and the cytokine combinations IL-12 with GM-CSF and IL-12 with IL-2 (32, 39–41). In the present work, we show the specific binding of the bi-AbFPs to HER2/*neu* expressed on the surface of tumor cells by flow cytometry. We have also shown binding of IL-12 and/or IL-2 AbFPs to the CT26 cell surface through their fused cytokines, a property described for the first time in AbFPs, and that is a consequence of the ability of IL-12 and IL-2 to bind heparin and other glycosaminoglycans (15, 45). Although GM-CSF is also known to bind cell surface glycosaminoglycans (47), we did not observe significant binding to CT26 cells. This may in part be due to the binding of GM-CSF to glycosaminoglycans in a pH-dependent manner, with maximum binding at pH 4.0 and no binding at pH $\geq$ 6.0 (46). Therefore, using conditions within the physiologic range (pH 7.4) is expected to result in negligible GM-CSF binding to glycosaminoglycans. Surprisingly, (IL-12)-IgG3-(IL-2)

Table 2. *In vivo* experiment 2: survival of mice challenged with D2F2/E2 and CT26-HER2/*neu* over a period of 134 days

Tumor model	Treatment	Median survival (d)	Long-term survivors (134 d)	*P**			
				(IL-12)-IgG3-(IL-2) vs	(IL-12)-IgG3 + IgG3-(IL-2) vs	(IL-12)-IgG3-(GM-CSF) vs	(IL-12)-IgG3 + IgG3-(GM-CSF) vs
D2F2/E2	PBS	22	0/8	<0.001↓	<0.001↓	<0.001↓	<0.001↓
	IgG3	23	0/8	<0.001↓	<0.001↓	<0.001↓	<0.001↓
	IgG3-(IL-2)	29	0/8	<0.001↓	<0.001↓	<0.001↓	<0.001↓
	IgG3-(GM-CSF)	84	2/8	0.489	0.130	0.097	<0.003↓
	(IL-12)-IgG3	134	5/8	0.813	0.443	0.586	0.064↓
	(IL-12)-IgG3 + IgG3-(IL-2)	134	6/8	0.173	NA †	0.703	0.114
	(IL-12)-IgG3 + IgG3-(GM-CSF)	134	8/8	<0.009↑	0.114	0.064↑	NA
	(IL-12)-IgG3-(IL-2)	109	3/8	NA	0.173	0.207	<0.009↓
	(IL-12)-IgG3-(GM-CSF)	134	5/8	0.207	0.703	NA	0.064↓
CT26-HER2/*neu*	PBS	16	0/8	<0.005↓	<0.001↓	<0.005↓	<0.001↓
	IgG3	18	0/8	<0001↓	<0.001↓	<0.001↓	<0.001↓
	IgG3-(IL-2)	22	0/8	<0.005↓	<0.001↓	<0.005↓	<0.001↓
	IgG3-(GM-CSF)	20	0/8	0.010↓	<0.001↓	<0.008↓	<0.001↓
	(IL-12)-IgG3	134	5/8	0.067↓	0.310	0.093	0.560
	(IL-12)-IgG3 + IgG3-(IL-2)	55	3/8	0.230	NA	0.204	0.111
	(IL-12)-IgG3 + IgG3-(GM-CSF)	134	6/8	0.018↑	0.111	0.017↑	NA
	(IL-12)-IgG3-(IL-2)	30	2/8	NA	0.230	0.905	0.018↓
	(IL-12)-IgG3-(GM-CSF)	28	2/8	0.905	0.204	NA	0.017↓

Abbreviation: NA, not applicable.

*The statistical evaluation of significant differences in survival between combinations of cytokines versus their respective controls was determined by Peto-Peto-Wilcoxon test. Arrows indicate significantly higher or lower survival at a $P < 0.07$.

†Indicate comparison between the same groups.

showed less binding to CT26 than (IL-12)-IgG3 despite containing two heparin-binding cytokines: IL-12 and IL-2. In addition, (IL-12)-IgG3-(GM-CSF) also showed a dramatic reduction in binding to CT26 compared with (IL-12)-IgG3. This apparent decrease in binding to CT26 exhibited by both bi-AbFPs suggests that the presence of IL-2 or GM-CSF at the COOH terminus of the heavy chain somehow decreases the heparin-binding activity of the IL-12 moiety fused at the NH_2 terminus of the heavy chain. This is somewhat unexpected, given that the cytokines were fused to opposite ends of the bi-AbFP heavy chain and were separated by the long hinge region of human IgG3 (34). An alternative explanation is that the presence of two cytokines fused to the same IgG3 heavy chain interferes with the binding of the secondary biotinylated antihuman polyclonal IgG/streptavidin-phycoerythrin complex used to detect the AbFP binding. However, we obtained a similar result when we used an antihuman κ monoclonal IgG1-FITC conjugate to detect the binding of the AbFPs (data not shown). Thus, although a reduction in signal could represent a byproduct of structural hindrance by the fused cytokines, the use of antibodies against two different epitopes on the AbFPs suggests instead a reduction in heparin binding by the cytokine moieties. Surprisingly, the suggested decrease of IL-12 heparin binding does not affect its ability to induce IFN-γ secretion by KY-1 cells. The latter observation was somewhat unexpected, because treatment of the KY-1 cell line with chondroitin ABCase to remove surface glycosaminoglycans results in a significant decrease of IL-12-induced IFN-γ secretion (18) and suggests that the ability of the cytokine to bind the surface glycosaminoglycans through its cytokine moiety is essential for inducing IFN-γ secretion. The ability of (IL-12)-IgG3-(IL-2) and (IL-12)-IgG3-(GM-CSF) to trigger IFN-γ secretion in KY-1 cells implies either that the decrease in heparin binding, if present, was not sufficient to affect IFN-γ secretion or that the interaction through the heparin binding site was not required for (IL-12)-IgG3 to trigger IFN-γ secretion. Finally, the binding of the bi-AbFPs to CT26-HER2/*neu* was only slightly reduced by incubation with heparin, confirming the ability of AbFPs to bind cells through direct interaction with HER2/*neu*.

IL-2 and IL-12 have been shown to enhance the cytolytic activity of NK cells (49), which are major effectors in the human innate resistance to cancer (50, 51). Consistent with previous studies showing that GM-CSF does not enhance cytolytic activity of NK cells (52), the NK cytotoxicity induced by (GM-CSF)-IgG3 was not significant. We have seen *in vitro* that the combination of (IL-12)-IgG3 and IgG3-(IL-2) induced greater cytotoxicity in splenocytes against

the NK-sensitive cell line YAC-1, than did (IL-12)-IgG3 or IgG3-(IL-2) alone, although with less than additive stimulation. These results are in agreement with previous studies, where a combination of (IL-12)-AbFP and (IL-2)-AbFP targeting CD30 showed an enhanced NK activation compared with the use of mono-AbFPs alone (53). However, this work did not include a comparison with a construct containing the two cytokines fused to one antibody (bi-AbFP). Surprisingly, the (IL-12)-IgG3-(IL-2) bi-AbFP did not enhance NK stimulation compared with IgG3-(IL-2) alone. The reason for the observed decrease in NK cytotoxicity elicited by the bi-AbFPs is unclear, but it is possible that the presence of IL-12 and IL-2 in the same molecule prevents the simultaneous binding to both cytokine receptors.

Previous studies exploring the *in vivo* therapeutic properties of an anti-EpCAM IgG1 IL-12/IL-2 bi-AbFP showed effective antitumor activity in a murine model of Lewis lung carcinoma when injected i.t., and to a lesser extent, when injected i.v. (54). However, it is also important to note that a bi-AbFP containing murine IL-12 and tumor necrosis factor-α fused to a single-chain Fv specific for the extracellular domain of B fibronectin was functional *in vitro* but showed modest antitumor activity *in vivo* against teratocarcinoma tumors compared with the corresponding mono-AbFPs (55). Previously, we have shown *in vivo* that combinations of anti-HER2/*neu* mono-AbFPs as well as bi-AbFPs were more effective than mono-AbFPs alone used as adjuvants of human extracellular domain of human HER2/*neu* protein prophylactic vaccination against TUBO cells, a murine breast cancer model (32). Progressive growth of TUBO cells in BALB/c mice give rise to lobular carcinoma, histologically similar to that seen in female BALB-*neu*T-transgenic mice (56). Although the TUBO cell line is a relevant tumor model in terms of pathophysiology of the disease, it expresses rat HER2/*neu* instead of human HER2/*neu* on its surface, which differs from human in nearly 10% of its amino acid sequence (57), facilitating extensive cross-reaction in the immune response. Because our AbFP does not cross-react with rat HER2/*neu* as shown by flow cytometry,[5] the use of these cells in a therapeutic setting to be targeted by our AbFPs is inapplicable. Instead, we used the epithelial syngeneic tumor models expressing human HER2/*neu* (D2F2/E2, CT26-HER2/*neu*, and MC38-HER2/*neu*) to allow appropriate tumor targeting.

Tumors that involve the peritoneal cavity are difficult to diagnose at early stages and at the time of detection are usually malignant, being metastatic tumors far more frequent than primary tumors (58). In female patients, ovarian tumor is the most frequent fatal cancer of the female reproductive tract in developed countries and a leading cause of cancer death for U.S. and European women (59). It is also the most common primary site of peritoneal carcinomatosis. Other tumors with potential to spread into the peritoneal cavity are primary tumors from stomach, colon, breast, bladder, and lung (58, 60). HER2/*neu* overexpression is observed in 10% to 30% of ovarian cancer cases and is associated with poor prognosis (6). HER2/*neu* gene amplification has been observed also in bladder, breast, colon, stomach, and lung cancers (61). Because these cancers are of epithelial origin, we injected i.p. murine epithelial tumor cells expressing human HER2/*neu* as *in vivo* models for primary or secondary peritoneal cancer.

The epithelial tumor models D2F2/E2, CT26-HER2/*neu* (both syngeneic for BALB/c), and MC38-HER2/*neu* (syngeneic for C57BL/6) were tested. The tumor model syngeneic for different mice strains was decided based on the tendency of BALB/c to elicit a Th1 response and of C57BL/6 to elicit a Th2 response (62). We first investigated the tumor protection elicited by (IL-12)-IgG3 and the bi-AbFPs after i.p. challenge with D2F2/E2, CT26-HER2/*neu*, or MC38-HER2/*neu.* (IL-12)-IgG3 showed significant protection, that was ≥50% long-term survivors in all three i.p. tumor models. However, protection elicited by i.p. treatment with (IL-12)-IgG3-(GM-CSF) and (IL-12)-IgG3-(IL-2) did not improve the protection by (IL-12)-IgG3 alone. In the case of D2F2/E2, protection generated by the bi-AbFPs was similar to (IL-12)-IgG3, MC38-HER2/*neu* was slightly lower but against CT26-HER2/*neu* was significantly lower compared with (IL-12)-IgG3. These results suggest that different types of effector mechanisms are required to elicit an optimal antitumor response in different tumor models expressing the same antigen. Studies using D2F2/E2 in a vaccination setting show that CTL and IFN-γ are both important for conferring tumor protection (63). Other studies using CT26-HER2/*neu* describe its unique sensitivity to NK- and T cell–based immunity (64). Finally, MC38 tumor immune rejection involves in part IFN-γ, $CD4^+$, and $CD8^+$ cells (65). Previous studies using an anti-GD2 chimeric human-mouse antibody fused to human IL-2 [ch14.18-(IL-2)] have shown that it can effectively eliminate tumor metastasis in syngeneic murine models of melanoma and neuroblastoma expressing the human antigen on the surface. In the melanoma model, the treatment with ch14.18-(IL-2) elicited a potent $CD8^+$ T cell–dependent immunity that was long-lasting and transferable (66–68). Treatment with the same AbFP induced a cell-mediated immunity that eradicated neuroblastoma metastasis and was completely NK dependent (69).

Many different mechanisms can be responsible of the immune protection elicited by our AbFPs. The cytokines IL-2, IL-12, and GM-CSF are known to be potent immunostimulators. IL-2 can stimulate T cells to proliferate and become cytotoxic (70), activate NK cells, and induce the lymphokine-activated killer cells (71). IL-12 promotes cell-mediated immunity (72) by inducing naive $CD4^+$ T cells to differentiate into Th1 cells (73), enhancing the cytotoxicity of NK and CTL cells (74), promoting the secretion of IFN-γ by T and NK cells that can retard tumor growth by inhibiting tumor angiogenesis (75) and up-regulated MHC class I expression (76). GM-CSF exhibits pleiotropic effects, including the augmentation of antigen presentation (77), increased expression of MHC class II on monocytes and

[5] Unpublished results.

macrophages and enhanced synthesis of adhesion molecules on granulocytes and monocytes (78). The antibody effector functions include antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity (79). Because the cytokines can activate NK cells, neutrophils, and macrophages, which are the effectors cells for antibody-dependent cellular cytotoxicity (79), their fusion with antibodies improve their independent antitumor effect (30). Protection may be elicited by the cytokine moiety, the antibody moiety, and by the combination of both, making it difficult to identify the individual role played by the different elements. Previous work in our laboratory showed that IgG3-(GM-CSF) could enhance Th1 and Th2 responses (41), whereas (IL-12)-IgG3 switched the immune response to Th1 (40). Hence, we expect that similar responses may be induced in the current setting. Another twist in the immunostimulation may be generated by bi-AbFPs and combination of mono-AbFPs, as can be seen from the different protection elicited in the tumor models used. Further studies are needed to elucidate the mechanisms involved in these complex responses.

To address whether the differential protection elicited by the combined use of cytokines is attributable to their simultaneous presence in the same AbFP, we investigated the protection elicited by IL-12/GM-CSF or IL-12/IL-2 as bi-AbFPs versus their corresponding combination of mono-AbFPs compared with the mono-AbFPs alone against D2F2/E2 and CT26-HER2/*neu*. Although treatment with our bi-AbFPs as direct therapeutics targeting HER2/*neu* expressing tumors resulted in a mixed response depending on the tumor model used, the combination of (IL-12)-IgG3 and IgG3-(GM-CSF) resulted in high protection in both tumor models tested, achieving 100% long-term survival in mice challenged with D2F2/E2 and 75% in mice challenged with CT26-HER2/*neu*. This combination resulted in higher levels of protection than any other treatment tested in these models. Is possible that the ability of mono-AbFP combinations to independently target two different cytokine receptors may circumvent detrimental limitations of the bi-AbFP structure and help protect with superior efficacy against different tumors. The fact that in the bi-AbFPs two different cytokines are fused to the same molecule may result in the sequestering of both cytokines by some cell populations expressing only one of the cytokine receptors, compromising the availability of the unbound cytokine to bind other effector cells. On the contrary, the cytokines in the combination of mono-AbFPs are readily available to target their receptors expressed in different effector cells and, in consequence, favor a more effective protection. In addition, it is possible that the apparently diminished heparin-binding activity of the bi-AbFPs may result in lower retention in the peritoneal cavity and faster removal from the tumor vicinity, reducing their efficacy. Although the reduced NK activation in the case of (IL-12)-IgG3-(IL-2) may be a factor that contribute to its lower performance, the combination of (IL-12)-IgG3 and IgG3-(GM-CSF) did not activate NK cells more than (IL-12)-IgG3 alone, and both groups were as good as or better than the bi-AbFP. Although the treatment with the AbFPs resulted in variable protection, most mice that achieved long-term survival also acquired immunologic memory that protected them after s.c. rechallenge. This response may be a product of an adjuvant effect by the AbFPs in close contact with tumor cells killed by a local innate immune response also favored by the AbFPs. These examples illustrate that different types of antitumor activity can be elicited against different tumors expressing the same antigen when treated with the same AbFP. Further studies are necessary to understand the mechanisms that are responsible of the immune protection elicited by the different treatments *in vivo*.

Studies with a DNA vaccine encoding wild-type human HER2/*neu* have shown protection against challenge with the D2F2/E2 tumor model. These long-term survivors have also shown 50% protection after subsequent challenge with the parental cell line not expressing HER2/*neu* (80). In this case, challenge with the HER2/*neu* expressing tumors may have resulted in priming of the immune system and broadening of epitope recognition mediated by $CD8^+$ T cells against D2F2 cells not expressing human HER2/*neu*. In our case, it is possible that the initial tumor challenge and the immediate therapeutic treatment with the AbFPs primed the immune system against the tumor cells. In addition, rechallenge with the HER2/*neu* expressing tumors may have boosted the immunity against the tumor cells, simultaneously extending the epitope recognition to other antigenic determinants on the surface of the tumor cells and resulting in the rejection of the parental cell line not expressing HER2/*neu*. To the best of our knowledge, this is the first time that combination of cytokines fused to antibodies either as mono-AbFPs or bi-AbFPs show systemic protection up to 4 months after the original challenge and epitope spreading. Further studies are necessary to elucidate the mechanisms behind this pattern of protection.

The levels of protection obtained against different murine tumor models by the combination of IL-12/GM-CSF mono-AbFPs and other AbFPs highlights their potential use to improve the management of peritoneal cancers expressing HER2/*neu*. The list of practical concerns associated with i.p. chemotherapy of epithelial tumors describes side effects, including peritoneal cavity infections and bowel perforation and obstruction (81). The potent antitumor activity of AbFP targeting HER2/*neu* in different types of epithelial peritoneal cancers promises more effective treatments with reduced side effects. The use of antibody-cytokine combinations to elicit a synergistic immune response takes antibody mediated targeting of HER2/*neu*–expressing tumors, a field pioneered by Herceptin, one step closer to a more complete anticancer immunotherapy. Changing the variable regions to target other tumor-associated antigens may further increase the versatility of AbFPs as direct antitumor therapeutics and help broaden the range of effective therapeutics against peritoneal carcinomatosis. Our results suggest that this approach will be relevant to patients with primary or metastatic peritoneal tumors of epithelial origin that may be observed in ovarian, colon, stomach, bladder, lung, and breast cancers.

Acknowledgments

We thank Dr. Wayne M. Yokohama for the KY-1 cell line; Dr. Wei-Zen Wei for the D2F2 and D2F2/E2 cell lines; Drs. Sherie Morrison, Tracy Daniels, and Koteswara Chintalacharuvu for their support and helpful suggestions on this article; and Vivian Kyinn for her skillful technical support.

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