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Generation of HER-2/neu-Specific Cytotoxic Neutrophils In Vivo: Efficient Arming of Neutrophils by Combined Administration of Granulocyte Colony-Stimulating Factor and Fcγ Receptor I Bispecific Antibodies

Ingmar A. F. M. Heijnen,*‡ Leonie J. M. Rijks,‡ Anja Schiel,§ Bernhard Stockmeyer,§ Heidi H. van Oijik,* Michael Dechant,§ Thomas Valerius,§ Tibor Keler,* Alison L. Tutt,† Martin J. Glennie,‖ Eric A. van Royen,‡ Peter J. A. Capel,* and Jan G. J. van de Winkel2*‡

Abstract

Ab-induced inflammatory antitumor responses by recruiting IgG Fc receptor (FcγR)-bearing cytotoxic effector cells. We recently described the capacity of the high affinity FcγRI (CD64) to trigger cytotoxic activity of neutrophils (PMN) during granulocyte colony-stimulating factor (G-CSF) treatment. To take advantage of FcγRI as a cytotoxic trigger molecule on PMN, two Ab constructs were prepared. We show that a chimeric human IgG1 Ab (Ch520C9) and an anti-FcγRI bispecific Ab (BsAb; 22x520C9), both directed to the proto-oncogene product HER-2/neu, interact with FcγRI. In addition, both Ab constructs mediate enhanced lysis of HER-2/neu-expressing tumor cells by G-CSF-primed PMN. However, engagement of FcγRI by Ch520C9 was inhibited by sera IgG, thereby abrogating the enhanced Ch520C9-mediated cytotoxicity. BsAb 22x520C9, which binds FcγRI outside the ligand binding domain, effectively recruits the cytotoxic potential of FcγRI on G-CSF-primed PMN regardless of the presence of human serum. These results indicate that under physiologic conditions, serum IgG impairs activation of FcγRI-mediated cytotoxicity by conventional antitumor Abs. The IgG blockade can be circumvented with anti-FcγRI BsAbs. Using human FcγRI transgenic mice we demonstrate that BsAb 22x520C9 is able to engage FcγRI in vivo. BsAb 22x520C9 injected i.v. was readily detected on circulating PMN of G-CSF-treated transgenic animals. In addition, we showed that PMN remain “armed” with BsAb 22x520C9 during migration to inflammatory sites, and that after isolation such PMN specifically lyse HER-2/neu-expressing tumor cells. These results point to the possibility of targeting anti-FcγRI BsAbs to G-CSF-primed PMN in vivo, endowing them with specific anti-tumor activity. The Journal of Immunology, 1997, 159: 5629–5639.

Attempts to exploit the ability of Abs to specifically target selected Ags on malignant cells have resulted in a large variety of Ab-based immunotherapeutic protocols. Numerous reports described the activity of rodent mAbs directed against tumor-associated Ags to mediate the destruction of cancer cells in vitro when combined with immune system components (such as complement proteins and cytotoxic effector cells) (1). In addition, mAbs have been shown to induce efficient and long term depletion of human cancer cells in xenografted animal models (2–4). However, the results of clinical trials testing murine mAbs as therapeutic agents in human patients have, in general, been disappointing (1). To our knowledge only one randomized trial showed a survival benefit and a reduced recurrence rate after mAb therapy (5). It should be noted that in this latter study patients underwent curative surgery and were free of residual tumor before mAb treatment (5). In contrast to the limited therapeutic efficacy, antitumor mAbs labeled with radioisotopes have been successfully used in human patients as diagnostic tools in tumor imaging (6, 7). In addition, unconjugated mAbs have been reported to saturate tumor antigenic sites within human tumors (8). The unsatisfactory clinical performances of murine mAbs may thus be attributable to inefficient recruitment of the host’s immune effector components, rather than an inability to reach target Ags. New Ab constructs are, therefore, being developed that exhibit a more optimal interaction with immune system effector mechanisms involved in Ab-induced tumor cell lysis.

One effort to improve the therapeutic potential of mAbs is to replace rodent sequences in the Ab construct by human IgG counterparts (9). Such chimeric or humanized mAbs exhibit reduced immunogenicity and extended serum half-life (10, 11). Importantly, the presence of a human Fcγ constant region of the proper subclass enhances the efficacy of the therapeutic molecules to mediate Ab-dependent cellular cytotoxicity (ADCC) by human cytotoxic effector cells (12, 13). ADCC is considered a major mechanism by which Abs...
exert their antitumor effects (1, 14). By binding to IgG Fc receptors (FcR) expressed on immune effector cells, Abs coated on target cells are able to induce cell-mediated target destruction (14).

Three classes of FcR for IgG, FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16), have been identified on leukocytes. In contrast to FcγRII and FcγRIII, FcγRI exhibits a high affinity for IgG, and effectively binds the Fc region of monoclonal human IgG1 and IgG3 (15). Neutrophils (PMN) represent the most populous FcγR-expressing leukocyte subset in the body, and their numbers can be further increased by application of granulocyte CSF (G-CSF) as a lineage-specific growth factor (16). Under normal conditions, PMN express the low affinity FcγRIIa and FcγRIIIb. In vivo in stimulation with G-CSF, however, they additionally express FcγRI (17). PMN have cytolytic potential against a large variety of tumor cells in vitro (18) and are known to play an active role in the rejection of malignant cells (19, 20). We have recently compared the capacities of the different FcγR classes to trigger ADCC by PMN and observed that FcγRI is the predominant cytotoxic trigger molecule on G-CSF-primed PMN (17, 21). Theoretically, expression of the high affinity FcγRI offers the possibility to link antitumor mAbs directly to G-CSF-primed PMN in the circulation, thereby generating target Ag-specific effector cells. Under physiologic conditions, however, binding of therapeutic mAbs to FcγRI may well be inhibited by high concentrations of endogenous IgG (14). The use of bispecific Abs (BsAb) comprising an anti-FcγRI mAb portion directed against an epitope outside the IgG binding site may overcome this inhibitory effect of endogenous IgG (22). To address this hypothesis, we generated a chimeric human IgG1 Ab and an anti-FcγRI BsAb with specificity for HER-2/neu.

HER-2-neu (or c-erbB2) is a proto-oncogene protein belonging to the epidermal growth factor receptor family of tyrosine kinases (23). Overexpression of HER-2/neu has been reported in a variety of human malignancies, including ~30% of breast and ovarian cancers, and high expression levels have been found associated with poor prognosis (24). HER-2/neu has been proposed to represent a suitable target for Ab-based immunotherapy, since its overexpression is relatively restricted to cancer cells (11, 25).

This study addresses two major questions concerning the exploitation of FcγRI as a cytotoxic trigger molecule. We first investigated the performance of chimeric human IgG1 anti-HER-2/neu and FcγRI-directed bispecific anti-HER-2/neu Abs in mediating ADCC in vitro and in vivo using neutrophils as effector cells. We then examined whether the most effective Ab construct could engage human FcγRI on PMN in vivo using a transgenic mouse model, thus generating HER-2/neu-specific PMN.

Materials and Methods

Cell lines

The human breast carcinoma cell line SK-BR-3, the Burkitt’s lymphoma cell line Raji, and human monocytic U937 cells were obtained from the American Type Culture Collection (Rockville, MD). The LANS human neuroblastoma cell line was provided by Dr. J.-L. Teillault (Institut Curie, Paris, France). Cells were maintained in RPMI 1640 medium (Life Technologies, Paisley, U.K.) supplemented with 10% FCS, 100 U/ml penicillin, streptomycin, and 4 mM t-glutamine. Adherent growing SK-BR-3 and LAN1 cells were harvested using trypsin-EDTA (Life Technologies, Gaithersburg, MD).

Abs and Ab constructs

The murine (m) mAbs against target cells used in this study are 520C9 (mIgG1) recognizing the extracellular domain of the proto-oncogene product HER-2/neu (Medarex, Ammandale, NJ) (26), F3.3 (mIgG1) directed against HLA class II (27), and 7A4 (mIgG3) against distal golgiangle (G2p2; provided by Dr. J.-L. Teillault). Murine anti-human FcγRI mAb 22 (mIgG1) and its humanized counterpart mAb H22 (human (h) IgG1), engineered by complementarity-determining region grafting (28), were obtained from Medarex. Anti-Gr-1 mAb RB6-8C5 was obtained from Pharmingen (San Diego, CA). Human IgG1 and IgG2, isolated from sera of patients with multiple myeloma, were obtained from Dr. A. Vlug (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). FITC-conjugated F(ab’2), of affinity-purified Abs to mouse or human IgG were obtained from Proteus Immunoresearch (San Francisco, CA).

BsAbs 22x520C9, H22x520C9, and 22xF3.3 were constructed by chemically cross-linking Fab’ of the respective parental Abs as previously described (29). Briefly, Abs were digested with papain to F(ab’2), which were reduced to Fab’ by 10 mM mercaptoethanolamine. The SH groups on one of the Fab’ partners were malimidated with excess o-phenylenediamine. After separation from free o-phenylenediamineimide on a Sephadex G-25 column, these Fab’-maleimide were combined with the other Fab’ partner at a molar ratio of 1:1 to generate Fab’ × Fab’ bspecific constructs. BsAbs were purified by size exclusion chromatography on Superdex 200 (Pharmacia, Piscataway, NJ), concentrated, and sterilized before use. The biochemical characteristics of the BsAbs are detailed elsewhere (25).

Chimeric Ch520C9 was prepared by chemically linking Fab’ of mAb 520C9 to human IgG1 Fc fragments exactly as previously described (30).

Mice

We previously described the generation of transgenic FVB/N mice carrying the human FcγRIA gene (31, 32). These mice express human FcγRI on monocytes and macrophages and at relatively low levels on neutrophils. Mice (2–4 mo old) of transgenic line 52 (32) were used throughout these experiments unless otherwise indicated. Mice were bred and maintained in the Transgenic Mouse Facility of the Central Laboratory Animal Institute (Utrecht University, The Netherlands) and were treated in accordance with institutional and national guidelines.

Cytokine treatment

Adult human individuals were treated with recombinant human G-CSF (r-hHuG-CSF, Neupogen; 3–5 μg/kg of body weight) from Amgen (Thousand Oaks, CA) for 5 to 10 days. All G-CSF-treated donors were included in clinical trials and served as a source for peripheral blood stem cell transplantation, approved by the ethical committee of the University Hospital Utrecht. In all cases, total white blood cell count was >3000/μl at the time of blood collection.

Mice were treated with recombinant mG-CSF (100 μg/kg of body weight; donated by Amgen) for 4 days unless otherwise indicated. The cytokine was administered once daily as an s.c. injection in 0.9% saline. Hematologic evaluation of mice was performed on peripheral blood obtained from the retro-orbital plexus. Total white blood cell counts were quantitated on a Coulter cytometer (Coulter Electronics, Luton, U.K.). Differential counts were determined on blood smears stained with Wright’s Giemsa.

Preparation of neutrophils

Peripheral blood (heparin-anticoagulated) was collected from control volunteers or from G-CSF-treated donors after informed consent was obtained. Neutrophils were isolated by Ficoll-Histopaque discontinuous gradient centrifugation as previously described (33). Contaminating erythrocytes were removed by hypotonic 0.2% NaCl lysis. PMN were >95% pure as determined by Cytoreps and >95% viable as checked by trypan blue exclusion.

**Mouse neutrophils.** Mouse neutrophils were isolated from the peritoneal cavity of transgenic and control mice as described previously (34). Mice that had been treated with rm-G-CSF were injected i.p. with 1 ml of 3% thioglycollate broth (Difco, Detroit, MI) to elicit peritoneal cells. Cells were harvested 4 h later by lavage of the peritoneal cavity with PBS containing 5 mM EDTA, resuspended in culture medium, and incubated in polystyrene flasks (Nunc, Roskilde, Denmark) at 37°C. After 1 h, nonadherent cells were harvested to separate neutrophils from adherent peritoneal macrophages. Cell suspensions contained >75% neutrophils, >15% monocytes, and <1% mast cells, as determined by flow cytometric analysis. For some experiments neutrophils were further enriched by magnetic cell sorting. Lymphocytes expressing CD4, CD8, and B220 were coated with MACS microbead-conjugated mAb GK1.5, 33-6.7, and RA3-6B2, respectively (Miltenyi Biotec, Bergisch Gladbach, Germany) and depleted using MACS microbead columns exactly as recommended by the manufacturer. Following this method, the purity of mouse neutrophils exceeded 90%.

**Flow cytometry**

Specific binding of Abs to effector and target cells was examined by flow cytometry. Cells (2 x 10⁶) resuspended in PBS containing 2.5% FCS and 0.05% sodium azide were incubated with Ab constructs (15 μg/ml) in the presence of various concentrations of human IgG or pooled human serum. Isolated PMN were first cultured for 2 h at 37°C in culture medium before Ab incubations to remove cytoplidal IgG. After 30 min on ice, cells were washed, and bound Ab was visualized by incubating the cells with FITC-labeled F(ab')₂ of anti-human or anti-mouse IgG. Cells were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). U937 cells cultured for 48 h in the presence of 250 U/ml IFN-γ (Genzyme, Cambridge, MA) were used to measure the binding characteristics of Ch520C9 and 22x520C9 to human FcyRI. Relative fluorescence intensity (RFI) was calculated as the ratio of mean fluorescence intensity (MFI) of irrelevant (isotype control) Ab to determine the in vivo binding of BsAb H22x520C9 to effector cells, whole blood samples or peritoneal exudate cells of mice injected i.v. with BsAb were stained with anti-human IgG-FITC and mAb Gr-1-PE, and analyzed by flow cytometry. Blood samples were treated with FACS Lysing Solution (Becton Dickinson), as recommended by the manufacturer, before flow cytometric analysis.

**Ab-dependent cellular cytotoxicity**

The cytolytic activity of human and mouse neutrophils was evaluated in a standard chromium release assay (17). Briefly, target tumor cells (1 x 10⁵) were labeled with 150 μCi of ⁵¹Cr (Amerham, Little Chalfont, U.K.) for 2 h at 37°C. After extensive washing, target cells were plated in U-bottom microtiter plates at a concentration of 5 x 10⁴ cells/well. Isolated human or mouse neutrophils were then added to each well, giving an ET/N ratio of 80:1. Cells were incubated at 37°C in the presence of various concentrations of Ab constructs in a final volume of 200 μl. In some experiments pooled human serum was added to the wells at a final concentration of 25%. For whole blood ADCC assays, 50 μl/well of anticoagulated peripheral blood was added instead of isolated neutrophils (21). After 4 h, ⁵¹Cr release was determined in the cell-free supernatants of duplicates. The percentage of cellular cytotoxicity was calculated according to the formula:

\[
\text{(% specific lysis)} = \left[\left(\text{experimental cmp} - \text{basal cmp}\right) / \left(\text{maximal cmp} - \text{basal cmp}\right)\right] \times 100
\]

where maximal ⁵¹Cr release determined after lysing target cells with 10% Zap-oglobin (Coulter), and basal release measured after incubating target cells with medium alone.

**Radiolabeling procedure**

Labeling of BsAb 22x520C9 with ⁹⁹ᵐTc was performed according to the method reported previously (35). First, 22x520C9 was conjugated to the bifunctional N-hydroxysuccinimide (NHS) ester of 6-[(4'-carboxy-phenyl)oxo]-1,2-dimercapto-2,1-dimethyl-4,8-diazaundecane (NHS-BAT ester) by mixing 1 mg of BsAb dissolved in 250 μl 0.1 M PBS (pH 8.5) with 15 μl of 4.5 mM NHS-BAT ester dissolved in dimethylformamide (DMF) at room temperature. The reaction was stopped by reducing the pH to 6.5 with 0.1 M KH₂PO₄. Unreacted NHS-BAT ester was removed by using Centricon 1 ultrafilter tubes (Sartorius, Göttingen, Germany) with a molecular mass cutoff of 20 kDa. The BAT ligand per Ab molecule ratio was determined as described previously (35) and ranged from 2.5 to 3.2. Then, 22x520C9 was radiolabeled by incubation for 5 min with a solution of 0.5 M NaCl, 0.1 M tartaric acid, followed by the addition of ⁹⁹ᵐTc-pertechnetate eluate and incubation for an additional 5 min. Purification of the ⁹⁹ᵐTc-labeled product was performed over a 1 ml Sephadex G-25 column in 0.9% saline. The ⁹⁹ᵐTc-labeled BsAb 22x520C9, which was obtained at a sp. act. of ~40 μCi/μg, was supplemented with unlabeled BsAb (ratio of ⁹⁹ᵐTc-labeled BsAb to unlabeled BsAb, 1:10), resulting in an sp. act. of 4 μCi/μg.

**Immunoscintigraphic imaging**

Transgenic mice of line 53 (32) and control littermates were used to study in vivo distribution of radiolabeled BsAb 22x520C9. Mice were anesthetized by injecting i.p. midazolam (Dormicum, Roche, Midrjedt, The Netherlands; 5 mg/kg of body weight) combined with a mixture of fluanisone and fentanyl citrate (Hypnorm, Janssen Pharmaceutica, Tilburg, The Netherlands; 0.25 mg/kg of body weight). Then, animals received i.v. injection of ~100 μCi of the ⁹⁹ᵐTc-labeled BsAb 22x520C9, which corresponded with 25 μg of BsAb via the tail vein. Immunoscintigraphy was conducted using a single-headed gamma camera (Diacam, Siemens Analytical X-Ray Instruments, Madison, WI) equipped with a low energy collimator with energy peak centered at 140 keV with a 10% window, and interfaced to a Sun work station (Hermes, Nuclear Diagnostics, Stockholm, Sweden). Immediately following injection of ⁹⁹ᵐTc-labeled 22x520C9, sequential anterior planar whole body images were recorded every minute for 30 min (matrix, 256 x 256 pixels; zoom factor, 2.5). At 30 min postinjection of ⁹⁹ᵐTc-BsAb, RL-8 (10 U/mouse; Genzyme) was injected s.c. in the right thigh to induce neutrophil migration. Sequential planar imaging was continued for another 30 min, and at 2 h after the injection of labeled BsAb, an anterior planar whole body image was recorded for 10 min. Quantification of the relative distribution of ⁹⁹ᵐTc-labeled BsAb was performed by region of interest (ROI) analysis on the whole body images. ROIs were drawn over the right and left thighs. Relative distribution was calculated by dividing the average counts per pixel within the area drawn over the right thigh by those within the area over the left thigh.

**Statistical analysis**

Statistical analyses were performed using unpaired Student’s t tests. Levels of significance are indicated in the text: p < 0.05 was considered significant.

**Results**

**Binding of chimeric and bispecific Abs to FcyRI and HER-2/neu**

Binding characteristics of chimeric human IgG1 anti-HER-2/neu and bispecific anti-FcyRI × anti-HER-2/neu Abs to FcyRI and HER-2/neu were examined. To allow direct comparison of chimeric and bispecific anti-HER-2/neu Abs, both Ab constructs were prepared from the same HER-2/neu-recognizing murine mAb 520C9 (26). Fab’ of this mAb were chemically conjugated either to human IgG1 Fc fragments (Ch520C9) or to Fab’ of the anti-FcyRI mAbs 22 (22x520C9) and H22 (H22x520C9). These latter anti-FcyRI mAbs recognize an epitope outside the IgG binding site, yet are able to trigger FcyRI effector functions (28, 36). mAb 22 and H22 have comparable binding characteristics but differ in backbone: mAb 22 is a murine IgG1, whereas humanized mAb H22 has a human IgG1 backbone (28). By taking advantage of the human IgG Fab’ portion of H22x520C9, this BsAb can be easily traced in mice (see below).

The specific binding of chimeric Ch520C9 and bispecific 22x520C9 and H22x520C9 to HER-2/neu-positive tumor cells and FcyRI-expressing effector cells was examined by flow cytometry. Indirect immunofluorescence demonstrated that both Ch520C9 and 22x520C9 (and H22x520C9) bind to HER-2/neu-expressing SK-BR-3 cells (Fig. 1), but not to HER-2/neu-deficient Raji or L4.1 cells (data not shown). When SK-BR-3 cells had been incubated with Ch520C9 or H22x520C9, the nonbinding human IgG portion could be readily detected (Fig. 1), demonstrating physical linkage of this portion to the HER-2/neu-binding murine portion. In addition, the Ab constructs Ch520C9, 22x520C9, and H22x520C9 were able to bind to FcyRI-expressing PMN isolated from G-CSF-treated donors. FITC-conjugated F(ab’)$_2$ of antimouse IgG were used to discriminate bound chimeric or bispecific Abs from possible residual human IgG bound to FcyR. Interestingly, when these experiments were performed in the presence of...
FIGURE 1. Binding of chimeric Ch520C9 and bispecific 22x520C9 and H22x520C9 Abs to FcyRI-expressing human PMN from G-CSF-treated donors and to HER-2/neu-positive SK-BR-3 breast cancer cells analyzed by flow cytometry. G-CSF-primed PMN were incubated with saturating concentrations (15 μg/ml) of Ch520C9, 22x520C9, or H22x520C9, and bound Ab constructs were revealed with Fab', anti-mouse IgG(F+L)-FITC (upper panels). Ab constructs Ch520C9 and H22x520C9 bound to SK-BR-3 cells were detected using Fab', anti-human IgG-FITC, and BsAb 22x520C9 was detected using Fab', anti-mouse IgG-FITC (lower panels).

![Flow cytometry images showing binding of Ab constructs to PMN and SK-BR-3 cells.](image)

FIGURE 2. Effects of human serum and human IgG on binding of chimeric Ch520C9 and bispecific 22x520C9 Abs to FcyRI. IFN-γ-treated U937 cells were incubated with 15 μg/ml Ch520C9 (open squares) or 22x520C9 (closed squares) in the presence of the indicated concentrations of pooled human serum (A) or purified human IgG (B). The binding of Ch520C9 and 22x520C9 was evaluated by flow cytometry and expressed as the MFI. The percent binding was calculated: [(MFI with competing serum or IgG - MFI control)/(MFI without competing serum or IgG - MFI control)] × 100%. Three independent experiments performed in duplicate produced similar results.

25% pooled human serum, binding of Ch520C9 to G-CSF-primed PMN was no longer detectable, whereas binding of 22x520C9 and H22x520C9 was unaltered. To further document the influence of human serum on binding of Ab constructs to FcyRI, IFN-γ-treated U937 cells that express high levels of FcyRI were used. Figure 2A illustrates that binding of Ch520C9 (15 μg/ml), but not 22x520C9, to FcyRI was inhibited in the presence of increasing amounts of human serum. Incubation in the presence of purified human IgG demonstrated a similar inhibitory effect, indicating that the blocking effect of human serum resides in the IgG fraction (Fig. 2B). Furthermore, purified human IgG1 (100 μg/ml) blocked Ch520C9 binding to FcyRI, whereas human IgG2, which does not bind to FcyRI (15), did not (data not shown). These data indicate that chimeric human IgG1 Abs bind to nonsaturated FcyRI in vitro, but are unable to efficiently engage this receptor under physiologic conditions.

**Chimeric and BsAb-mediated lysis of tumor cells by PMN**

We next evaluated the antitumor effects of chimeric Ch520C9 and bispecific 22x520C9 against SK-BR-3 cells using PMN effectors. In the presence of Ch520C9, an Ab dose-dependent tumor cell killing was observed with PMN from both control and G-CSF-treated donors (Fig. 3, A and B). However, at the concentrations of Ch520C9 tested (ranging from 0.08-10 μg/ml), G-CSF-primed PMN were much more effective in mediating ADCC than PMN from control donors (Fig. 3, A and B). Because Ch520C9 is able to engage FcyRI, these data reflect the altered phenotype of PMN during G-CSF therapy (i.e., increased FcyRI expression (17)) and support observations indicating that FcyRI represents an effective trigger molecule on PMN from G-CSF-treated donors (17, 21, 27). When ADCC experiments were performed in the presence of 25% human serum, no significant (p > 0.05) change in Ch520C9-mediated tumor cell killing by control PMN was observed (Fig. 3, A and C). This is in line with the finding that low affinity FcyRII/III are present on control PMN (15); engagement of these receptors by Ch520C9 should not be hindered by serum IgG. Notably, addition of human serum (25%) to PMN from G-CSF-treated donors resulted in a strong decrease in Ch520C9-mediated cytotoxicity (Fig. 3, B and D). Under these conditions, the capacity of G-CSF-primed PMN to induce tumor cell lysis was comparable to that of control PMN (Fig. 3, A and D). These results show that the impaired engagement of FcyRI in the presence of serum IgG by the chimeric anti-HER-2/neu Ab (as seen in Fig. 2) severely limits functional performance of the Ab (Fig. 3).

To assess whether FcyRI and its capacity to trigger ADCC by G-CSF-primed PMN could be recruited in the presence of human serum in a distinct way, bispecific 22x520C9 was tested. Figure 3B shows that the effect of bispecific 22x520C9 on target cell killing by G-CSF-primed PMN was comparable to that of chimeric
FIGURE 3. Analysis of chimeric Ch520C9 and bispecific 22x520C9 Ab-mediated cytotoxicity. Cytotoxicity by human neutrophils from healthy donors (HD; A and C) or from G-CSF treated donors (G-CSF; B and D) against HER-2/neu-targeted SK-BR-3 breast cancer cells, mediated via Ch520C9 (open squares) or 22x520C9 (closed squares), was compared. ADCC assays were performed in the absence (A and B) or the presence (C and D) of pooled human serum (25%) to evaluate the inhibitory effect of serum IgG on Ch520C9 and 22x520C9 Ab-mediated ADCC by PMN. General conditions for ADCC are described in Materials and Methods. Results are presented as the mean ± SEM of the percent specific lysis of three separate experiments with different donors. * indicates p < 0.05 (Ch520C9-mediated ADCC vs 22x520C9-mediated ADCC).

FIGURE 4. Ab-mediated whole blood cytotoxicity against SK-BR-3 target cells. Citrate anticoagulated whole blood from healthy donors (HD; A) or from G-CSF-treated donors (C-CSF; B) was used as an effector source for chimeric Ch520C9 (open squares) or bispecific 22x520C9 (closed squares) Ab-mediated ADCC. Results from three independent experiments are presented as the mean ± SEM percent specific lysis. * indicates p < 0.05 (Ch520C9-mediated ADCC vs 22x520C9-mediated ADCC).

Ch520C9, albeit high concentrations of BsAb (10 µg/ml) were less effective than chimeric Ab. In sharp contrast to Ch520C9-mediated cytotoxicity, however, human serum (25%) did not reduce 22x520C9-mediated target cell killing by G-CSF-stimulated PMN (Fig. 3, B and D). Thus, in the presence of human serum, the combination of BsAb 22x520C9 with neutrophils from G-CSF-treated donors was most effective in inducing lysis of HER-2/neu-expressing targets. As expected, PMN from control donors did not lyse target cells in the presence of bispecific 22x520C9 (Fig. 3, A and C), since these PMN do not sufficiently express FcyRI (17). When SK-BR-3 tumor cells were incubated with chimeric Ch520C9 or bispecific 22x520C9 Abs in combination with human serum alone, no lysis was detected (not shown; n = 4), indicating that complement-mediated lysis was not induced by these Ab constructs.

To further evaluate Ab efficacy under conditions reflecting a physiologic situation, whole blood ADCC experiments were performed. Minimal or no detectable killing with Ch520C9 or 22x520C9, respectively, was observed using whole blood from control donors (Fig. 4A). Enhanced levels of Ch520C9-mediated killing were obtained with whole blood from G-CSF-treated donors (Fig. 4B), probably due to increased PMN numbers. Using whole blood from G-CSF donors, BsAb 22x520C9 induced killing of SK-BR-3 cells efficiently and more potently than Ch520C9, especially at low Ab concentrations (53.9 ± 9.6 and 76.3 ± 6.5% specific lysis at 0.08 and 0.4 µg/ml 22x520C9, respectively, vs 20.3 ± 3.3 and 30.2 ± 3.4% for Ch520C9). Collectively, these data suggest that engagement of FcyRI via its ligand binding site is impaired by serum IgG under physiologic conditions, and that anti-FcyRI bispecific Abs may overcome this IgG blockade.
To investigate whether anti-FcyRI BsAbs are able to engage transgenic mice as a model. We previously showed that these mice FcyRI on G-CSF-primed PMN in vivo, we used human FcyRI from transgenic mice receiving rmG-CSF (100 pg/kg/day) for 4 days, as described in Materials and Methods, and were incubated with human SK-BR-3 (closed bars), Raji (open bars), or LAN1 (hatched bars) tumor cells at an E:T ratio of 80:1 in the presence of 1 pg/ml BsAb 22x520C9, 22xF3.3, and/or mAb 7A4, as detailed below the graph. One representative experiment of three performed is shown.

Table 1. Effect of rmG-CSF on total leukocyte count, differential counts, and PMN hFcyRI expression in transgenic mice

<table>
<thead>
<tr>
<th>Days after Treatment</th>
<th>White Blood Cell Count</th>
<th>Differential Count</th>
<th>hFcyRI Expression on PMN</th>
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</thead>
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<tr>
<td>0</td>
<td>7.4 ± 1.5</td>
<td>90.7 ± 2.5 (6.7)</td>
<td>8.3 ± 2.1 (0.3)</td>
</tr>
<tr>
<td>1</td>
<td>9.8 ± 2.9</td>
<td>73.5 ± 3.2 (7.0)</td>
<td>22.8 ± 4.5 (2.2)</td>
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<tr>
<td>2</td>
<td>11.7 ± 3.3</td>
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<td>37.0 ± 6.2 (4.4)</td>
</tr>
<tr>
<td>3</td>
<td>26.2 ± 2.4</td>
<td>49.3 ± 2.5 (12.9)</td>
<td>44.7 ± 1.6 (11.7)</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td>2.3 ± 0.8 (0.5)</td>
</tr>
</tbody>
</table>

* Human FcyRI transgenic mice (n = 5) were injected s.c. with rmG-CSF (100 µg/kg body weight) once daily. Blood was analyzed 18 h after injection.
* White blood cell count given X 10^6/ml.
* Differential counts given in percentages of total white blood cells. Numbers in parentheses represent absolute numbers (X 10^6/ml) calculated by multiplying the total white blood cell count with the respective differential count.
* Expression of human FcyRI on PMN given in RFI (see Materials and Methods).
* Day 0 = before treatment.

FIGURE 5. Human FcyRI-mediated lysis of human tumor cells by peritoneal neutrophils of transgenic mice. Neutrophils were isolated from transgenic mice receiving rmG-CSF (100 µg/kg/day) for 4 days, as described in Materials and Methods, and were incubated with human SK-BR-3 (closed bars), Raji (open bars), or LAN1 (hatched bars) tumor cells at an E:T ratio of 80:1 in the presence of 1 µg/ml BsAb 22x520C9, 22xF3.3, and/or mAb 7A4, as detailed below the graph. One representative experiment of three performed is shown.

FcyRI-directed BsAbs mediate ADCC by PMN from human FcyRI transgenic mice treated with G-CSF

To investigate whether anti-FcyRI BsAbs are able to engage FcyRI on G-CSF-primed PMN in vivo, we used human FcyRI transgenic mice as a model. We previously showed that these mice selectively express human FcyRI on myeloid cells (31, 32). The human receptor associates with the murine signal-transducing FcR γ-chain, thereby constituting a functional receptor complex (37). Injection of transgenic mice with human G-CSF strongly up-regulates hFcyRI levels on circulating neutrophils (32). Here, we first evaluated the effects of murine G-CSF on PMN in more detail, and then the ability of PMN to lyse tumor cells via hFcyRI. Treatment of mice with rmG-CSF (100 µg/kg/day) induced an increase in total white blood cell counts that became pronounced after 2 to 3 days of treatment (Table I). Considering the white blood cell subsets, we observed that the elevation of total white blood cell numbers was mainly due to an absolute increase in neutrophils. Absolute neutrophil counts increased from 500 to 11,700 cells/µl (23×) after 4 days of rmG-CSF therapy. No differences were observed between transgenic mice and nontransgenic littermates (not shown). Human FcyRI expression on PMN increased substantially after 1 day of treatment, and maximal expression plateaued after 2 to 3 days of rmG-CSF administration (Table I).

Next, we tested whether triggering of hFcyRI on PMN from G-CSF-treated transgenic mice induces ADCC. As shown in Figure 5, PMN isolated from the peritoneal cavity of rmG-CSF-treated transgenic mice killed SK-BR-3 tumor cells in the presence of exogenously added BsAb 22x520C9, which specifically recruits PMN via hFcyRI. HER-2/neu-negative Raji and LAN1 target cells were not lysed via BsAb 22x520C9. However, specific lysis of HLA class II-expressing Raji cells was observed when G-CSF-primed transgenic PMN had been sensitized in vitro with 1.0 µg/ml BsAb 22xF3.3 (anti-hFcyRI × anti-HLA class II). PMN from G-CSF-treated nontransgenic control animals did not exhibit target cell lysis via BsAbs 22x520C9 and 22xF3.3 (not shown). In the presence of anti-G,, mAb 7A4 (mlG3; 1.0 µg/ml), LAN1 cells were killed by G-CSF-primed transgenic PMN, but not by PMN from nontransgenic mice, indicating that 7A4-mediated cytotoxicity is apparently triggered by hFcyRI. These data are in agreement with subclass specificity studies that showed that mlG3 binds with high affinity to hFcyRI, but not to mFcyRs (38). Interestingly, BsAb 22x520C9-sensitized PMN from G-CSF-treated transgenic mice lysed LAN1 cells in the presence of mAb 7A4 (Fig. 5). This demonstrates that hFcyRI can be engaged by its ligand binding site despite the bound anti-hFcyRI BsAb.

BsAb 22x520C9 binds to and relocalizes with G-CSF-primed PMN in vivo

The ability of anti-HER-2/neu BsAbs to engage human FcyRI on PMN in vivo was examined in transgenic mice treated with rmG-CSF. To induce maximal hFcyRI expression levels in transgenic mice, animals were treated with rmG-CSF for 4 days. On day 4, a single dose of H22x520C9 (25 µg/mouse) was infused i.v. Figure 6A shows the presence of BsAb H22x520C9 on the surface of circulating PMN in the blood of G-CSF-treated transgenic mice 2 h after BsAb infusion. No H22x520C9 could be detected on the surface of PMN from nontransgenic animals (Fig. 6A).

For therapeutic options, it is of importance to establish whether circulating PMN that have bound BsAb, remain
FIGURE 6. In vivo binding of bispecific Ab H22x520C9 to neutrophils from G-CSF-treated transgenic mice expressing human FcγRI. Nontransgenic and transgenic mice received rmG-CSF for 4 days and were then injected i.v. with a single dose of BsAb H22x520C9 (25 μg/mouse). A, Peripheral blood was isolated 2 h after BsAb injection and stained with PE-labeled Gr-1 mAb (anti-granulocyte) and with F(ab')2 anti-human IgG-FITC to detect bound BsAb. B, Inflammatory neutrophils were isolated from the peritoneal cavity of mice 5 h after BsAb injection (see Materials and Methods) and stained with Gr-1-PE and anti-human IgG-FITC. Samples were analyzed by flow cytometry, and data from one representative experiment are shown as fluorescence dot plots.

“armed” with the BsAb after migration from the microcirculation to sites of (antitumor) inflammation. Therefore, 1 h after BsAb infusion, an acute inflammatory response was induced in the peritoneal cavity of G-CSF-treated mice. A single i.p. injection of thioglycolate resulted within several hours in an accumulation of PMN in the peritoneal cavity. It has been well documented that these inflammatory PMN are derived from the circulation and marginate from small venules and capillaries (39). Four hours after the inflammatory response was initiated, PMN were isolated from the peritoneal cavity and subjected to FACS analysis to determine BsAb H22x520C9 binding. In contrast to inflammatory PMN from G-CSF-treated control mice, G-CSF-primed transgenic neutrophils were coated with substantial amounts of BsAb H22x520C9 (Fig. 6B).

We noted that the presence of BsAb 22x520C9 on the surface of inflammatory PMN in the peritoneal cavity could be due to high systemic levels of BsAb rather than actually comigrating with these PMN from the circulation. To exclude this possibility we conducted a series of scintigraphic imaging studies. Transgenic and control mice were treated with G-CSF for 4 days and then received i.v. a single dose (25 μg/mouse) of 99mTc-radiolabeled BsAb 22x520C9. To initiate a local inflammatory response, IL-8 was used. This cytokine has been shown to be chemotactic for neutrophils in vitro and to induce their accumulation in vivo at sites of inflammation (40). Therefore, 30 min after injection of 99mTc-labeled BsAb 22x520C9, one dose of IL-8 (10 U/mouse) was injected s.c. in the right thigh of mice. Radioimmunoscintigrams obtained at two different time points post-IL-8 injection are depicted in Figure 7. Five minutes after administration of IL-8, a small difference in the biodistribution of 99mTc-BsAb was observed between transgenic and nontransgenic mice. At this time, most of the radioactivity in mice was found in the blood pool and blood-rich organs, while a substantial part was localized in the liver, kidneys, and bladder. In addition, a small accumulation of 99mTc-22x520C9 activity was observed in the right thigh of transgenic mice, which was not seen in control animals (Fig. 7A). Images obtained 15 min later showed a further accumulation of labeled BsAb 22x520C9 only in the right thigh of transgenic animals (ROI ratios, 5.3 ± 2.0 for transgenic mice vs 1.6 ± 1.0 for controls; n = 3; Fig. 7B). The observed local radioactivity was temporary and vanished within 5 h (data not shown). The minor increase in radioactivity that was seen in the right thigh of control mice (Fig. 7B; ROI ratio, 1.6 ± 1.0) was probably due to local extravasation, indicating that the differences between transgenic and control mice were not due to absent IL-8-induced inflammatory events in controls. Thus, these results suggested that anti-hFcγRI BsAb 22x520C9 binds to G-CSF-stimulated PMN.
in vivo and remains bound to these immune cells during their migration.

In vivo generation of HER-2/neu-specific cytotoxic PMN

We next evaluated the effects of in vivo arming of PMN with BsAb 22x520C9 on their cytotoxic capacities. Transgenic and nontransgenic mice were treated with G-CSF for 4 days and received a single i.v. dose of BsAb 22x520C9 (10 μg/mouse) on day 4. Three hours after BsAb injection, whole blood was isolated and tested in ADCC experiments against target tumor cells. Whole blood drawn from transgenic mice that had been treated with both G-CSF and BsAb 22x520C9 lysed SK-BR-3 tumor cells without any further addition (Fig. 8A). No specific killing was observed when whole blood from nontransgenic animals was used (Fig. 8A), indicating that hFcyRI is essential for this process. Notably, we observed by light microscopy that SK-BR-3 cells that were incubated with whole blood from G-CSF/BsAb-treated transgenic mice were surrounded by murine leukocytes (Fig. 8B). Wright’s Giemsa staining revealed that these attached leukocytes primarily represent neutrophils. This phenomenon was not seen upon incubation with blood from G-CSF/BsAb-treated control mice (Fig. 8C). HER-2/neu lacking Raji and LAN1 cells were not killed by whole blood from transgenic mice treated with both G-CSF and BsAb 22x520C9. However, when mAb F3.3 was added exogenously, specific killing of Raji cells was detected, demonstrating that Raji cells can be killed under these conditions, probably via mouse FcyR (data not shown). We also noted that whole blood from transgenic animals receiving either G-CSF or BsAb alone did not exhibit specific

**FIGURE 7.** Whole body immunoscintigraphic images of hFcyRI transgenic and nontransgenic control mice treated with rmG-CSF and 99mTc-labeled BsAb 22x520C9 (25 μg ~ 100 μCi, on day 4) and given s.c. injections of IL-8 (10 U/mouse) in the right thigh to induce local neutrophil migration and accumulation. Ventral images recorded 5 min (A) and 20 min (B) after IL-8 injection of transgenic (left) and control (right) mice are shown. The arrowhead points to the site of intense IL-8-induced accumulation of radiolabeled BsAb.

**FIGURE 8.** Cytolysis of SK-BR-3 target cells by whole blood from hFcyRI transgenic mice receiving both G-CSF and BsAb 22x520C9. A, Whole blood ADCC against SK-BR-3 cells using heparin anticoagulated blood from transgenic (hatched bars) or nontransgenic (open bars) mice. Before blood was collected, mice had received either rmG-CSF or BsAb 22x520C9 treatment alone or had received a combination of G-CSF and BsAb, as indicated. Results from three independent experiments are presented as the mean ± SEM percent specific lysis. Blood from G-CSF/BsAb-treated transgenic mice mediated significant (* indicates p < 0.05) ADCC. During incubation of SK-BR-3 tumor cells with whole blood from transgenic mice treated with both G-CSF and BsAb 22x520C9, SK-BR-3 cells (large cells) were clustered with white blood cells (B). This phenomenon was not observed using whole blood from G-CSF/BsAb-treated nontransgenic animals (C). Photomicrographs were taken 2 h after the initiation of whole blood ADCC assays.
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SK-BR-3 cell lysis (Fig. 8A). The data showing absent killing in blood from mice without G-CSF administration are consistent with those obtained with human blood from control donors (Fig. 2) and support the idea that increased PMN numbers and up-regulated hFcyRI expression levels are responsible for enhanced ADCC. Indeed, when transgenic mice were treated for only 2 days with G-CSF before receiving 22x520C9, specific lysis of SK-BR-3 cells was about half that observed after 4 days of G-CSF therapy (not shown).

To demonstrate that the HER-2/neu-specific cytolytic activity results from in vivo arming of G-CSF-primed PMN, isolated PMN from mice were tested in ADCC. Inflammatory PMN killed SK-BR-3 target cells when they were isolated from the peritoneal cavity of transgenic mice that had received a combination of G-CSF and BsAb 22x520C9 as described in Materials and Methods. PMN were incubated with SK-BR-3 (closed bars), Raji (open bars), or LAN1 (hatched bars) tumor cells in the absence or the presence of 2.5 μg/ml anti-HLA class II mAb F3.3 or anti-GD2 mAb 7A4, as indicated.

Discussion

To elicit host antitumor inflammatory responses, Abs should not only bind to tumor cells, but also effectively recruit immune effector mechanisms. Several lines of evidence support the importance of FcyR-expressing cytotoxic effectors on the therapeutic outcome of Ab treatment. 1) Infiltration of FcyRI-bearing cytotoxic cells (i.e., macrophages) into tumor tissue has been correlated with mAb-induced rejection of transplanted tumors (41, 42). 2) FcyRIII-deficient mice are unable to reject tumors following mAb therapy (43). 3) The ability of mAbs with the same tumor specificity, but of different isotypes, to engage FcyRs and induce ADCC in vitro correlates with tumor cell depletion in mouse models (44). Considering the latter point, chimeric Abs with human Fcy constant regions have been developed to reduce the chance of human anti-mouse Ab (HAMA) formation and to prolong Ab presence in the circulation. However, the major benefit of chimeric Abs may be their capacity to effectively activate host effector mechanisms for antitumor responses. Of the four different human IgG subclasses, Abs with the human IgG1 constant part seem to be optimal for effector cell recruitment and ADCC (45).

The first goal of the present study was to evaluate the abilities of two different anti-HER-2/neu Ab constructs to engage FcyRI and to trigger FcyRI-mediated ADCC. Chimeric Ch520C9 was constructed by chemically linking the Fab' portion of HER-2/neu-recognizing mAb 520C9 to human Fcy1 constant regions, whereas bispecific 22x520C9 consisted of 520C9 Fab' linked to Fab' of anti-FcylR mAb 22 (or H22). Using PMN from control individuals and from G-CSF-treated donors, which differ in FcylR (CD64) expression (17), we were able to establish the efficacy of chimeric Ch520C9 to recruit the cytotoxic potential of FcyRI. This study demonstrates that compared with control PMN, PMN from G-CSF-treated donors exhibit enhanced lysis of SK-BR-3 tumor cells in the presence of Ch520C9. It has been reported that FcylR-mediated ADCC is not influenced by G-CSF (23), and that FcyRIIb does not mediate killing with isolated PMN (control and G-CSF-stimulated) (23, 29). Therefore, the enhanced killing by G-CSF-primed PMN in the presence of Ch520C9 can be attributed to FcylR. Importantly, this contention is supported by the observation that the differences in Ch520C9-mediated tumoricidal activity between control and G-CSF-primed PMN were not significant in the presence of human serum. In the light of these observations, it is important to note that whole human serum, polyclonal IgG, and monomeric human IgG1 (but not IgG2) inhibit binding of Ch520C9 to FcylR. These data support previous findings that FcylR represents a potent cytotoxic trigger molecule and suggest that engagement of this receptor in vivo by chimeric human IgG1 mAbs is impaired by endogenous IgG, thereby precluding the efficient use of FcylR as trigger molecule for ADCC. In addition, these findings argue that Ab-mediated activities not only should be examined with isolated cells, but also in the presence of competing serum IgG.

To improve FcylR recruitment, bispecific 22x520C9 was developed. This construct contains Fab' fragments of mAb 22 that react with FcylR at an epitope distinct from the ligand binding site and should thus engage FcylR regardless of the presence of serum IgG. Indeed, neither binding to FcylR by 22x520C9 nor its ability to mediate ADCC by G-CSF-primed PMN was affected by human serum or purified IgG. As expected, FcylR-deficient PMN isolated from control donors did not kill SK-BR-3 target cells via BsAb 22x520C9. Chimeric Ch520C9 and bispecific 22x520C9 were equally active in mediating ADCC by G-CSF-primed PMN, albeit high concentrations of 22x520C9 negatively influenced its activity. Saturation of both FcylR and HER-2/neu by 22x520C9, thereby impairing efficient linkage of the target and effector cells, probably underlies this effect. In the presence of human serum, however, BsAb 22x520C9 induced more potent ADCC by G-CSF-treated individuals as an effector source (Fig. 4). Neither Ch520C9 nor 22x520C9 induced...
significant levels of target cell lysis in the presence of whole blood from control donors. Taking into account that human serum does not significantly inhibit Ch520C9-mediated ADCC by control PMN, we believe that increased PMN numbers underlie the enhanced Ch520C9-mediated lysis observed in the presence of whole blood from G-CSF-treated individuals. Recently, Ely et al. demonstrated anti-FcγRI BsAb-dependent phagocytosis by macrophages in the presence of autologous serum and speculated that the BsAb was able to circumvent the inhibitory effect of serum IgG (46). Our results, however, are the first that directly compare a BsAb with an mAb and show that the activity of a mAb is indeed affected by serum IgG, in contrast to that of a BsAb.

Several advantages of using anti-FcγRI BsAbs, instead of conventional mAbs can be mentioned. 1) BsAbs selectively engage FcγRI. By this means, activation of undesired FcγR-mediated effects (e.g., down-regulatory effects triggered by FcγRlb (47)) and recruitment of noncytotoxic cells that express FcγR (e.g., B cells and platelets) are avoided. 2) Anti-FcγRI BsAbs are able to circumvent competition by human IgG and to maintain ADCC activity under serum conditions. 3) Binding of BsAb 22x520C9 to FcγRI does not occupy the ligand binding site, suggesting that IgG may still mediate its function by engaging FcγRI. These latter two possibilities were examined using a human FcγRI transgenic mouse strain. It was previously reported that these mice represent a relevant model for evaluation of FcγRI function in vivo (31, 32). We here show that these mice respond to mG-CSF similarly as humans do to h-G-CSF; i.e., both PMN numbers in the circulation and hFcγRI expression on PMN are enhanced (Table I). In addition, hFcγRI was active in mediating ADCC by transgenic mouse effector cells. Human SK-BR-3 cells were lysed by PMN from G-CSF-treated mice expressing tumor cells (Fig. 4). This finding was recently supported by the observation that FcγRI can mediate phagocytosis of mlgG3-coated Candida albicans regardless of occupancy with BsAb 22x520C9 (Dr. I. van den Herik-Oudijk, unpublished observations). After infusing BsAb 22x520C9 in G-CSF-treated transgenic animals, BsAbs could be readily detected on the surface of circulating and inflammatory PMN. During migration and extravasation of PMN from the circulation to surrounding tissue, we found that BsAb 22x520C9 remained bound to PMN. Moreover, PMN isolated from transgenic mice that had been treated with G-CSF and BsAb 22x520C9 selectively killed HER-2/neu-expressing tumor cells. Collectively, our findings demonstrate that HER-2/neu-specific cytotoxic PMN can be generated in vivo by combination administration of G-CSF and anti-FcγRI BsAbs.

Earlier work showed large infiltrates of PMN at tumor sites (48, 49). Since anti-FcγRI BsAbs bound to G-CSF-primed PMN endowed these unspecific effector cells with specific antitumor activity, we speculate thatarming of FcγRI-expressing effector cells might augment their tumoricidal activity in vivo and, thus, the therapeutic efficacy of the BsAb. Because clinical application of BsAbs composed of murine Fab′ is limited by their immunogenicity (25, 50), less immunogenic BsAbs are being developed. For example, the partly homogenized bispecific construct H22x520C9 comprising Fab′ of anti-FcγRI mAb H22 showed in vitro cytotoxic activity similar to that of BsAb 22x520C9 (see Footnote 4). Clinical trials testing BsAb H22x520C9 (designated MDX-H210) in combination with G-CSF are currently ongoing with encouraging results (51, 52). Based on the present data, we hypothesize that carefully chosen combinations of Ab constructs with cytokines may result in a more optimal clinical efficacy than generally obtained with conventional mAbs alone.

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