Bispecific Minibodies Targeting HER2/neu and CD16 Exhibit Improved Tumor Lysis When Placed in a Divalent Tumor Antigen Binding Format

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Lillian S. Shahied‡, Yong Tang, R. Katherine Alpaugh, Robert Somer, Dana Greenspon, and Louis M. Weiner§

From the Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

Unconjugated monoclonal antibodies have emerged as important therapeutic agents for selected malignancies. One mechanism by which antibodies can exert cytotoxic effects is antibody-dependent cellular cytotoxicity (ADCC). In an effort to increase the efficiency of ADCC at tumor sites, we have focused on the construction of bispecific antibodies specific for the tumor antigen HER2/neu and the FcγRIII-activating receptor (CD16) found on NK cells, mononuclear phagocytes, and neutrophils. Here, we describe the production of bispecific minibodies in two distinct binding formats. The parent minibody was constructed such that the IgG1 CH3 constant domain serves as the oligomerization domain and is attached to an anti-CD16 and an anti-HER2/neu single-chain Fv via 19- and 29-amino acid linkers, respectively. This molecule can be expressed in mammalian cells from a dicistronic vector and has been purified using sequential affinity purification techniques. Analysis by surface plasmon resonance shows that the bispecific minibody can bind to HER2/neu and CD16, both individually and simultaneously. Furthermore, cytotoxicity studies show that the minibody can induce significant tumor cell lysis at a concentration as low as 20 nM. A trimeric, bispecific minibody (TriBi) that binds dimerically to HER2/neu and monomerically to CD16 induces equivalent cytotoxicity at lower antibody concentrations than either the parent minibody or the corresponding single-chain dimer. Both minibody constructs are stable in mouse and human serum for up to 72 h at 37 °C. These minibodies have the potential to target solid tumors and promote tumor lysis by natural killer cells and mononuclear phagocytes.

Antibodies have emerged as important components of effective therapies for an increasing number of human malignancies. Unconjugated antibodies directed against the B-cell idiotype (1), CD20 (2, 3), and CD22 (4) exhibit significant utility in the therapy of lymphomas. One anti-CD20 antibody has become a widely used, Food and Drug Administration-approved agent with potential applications to other malignancies as well. Radioimmunoconjugates have also been constructed for imaging as well as therapy. Two agents that recognize CD20 have been shown to exhibit significant anti-tumor activity (5, 6) and have entered standard clinical practice for the treatment of lymphoma. An anti-CD52 antibody that efficiently mediates complement fixation has been approved for use in chemotherapy-refractory chronic lymphocytic leukemia (7). In addition, an immunoconjugate consisting of an anti-CD33 antibody and calicheamycin has been approved for use in refractory acute myeloid leukemia (8). Immunotoxins consisting of recombinant antibody fragments and catalytic toxins demonstrate anti-tumor activity as well (9). Other examples of antibodies that have shown therapeutic promise or have already been approved for use as therapeutic agents include an unconjugated anti-HER2/neu antibody that is widely used alone and in combination with chemotherapy agents in breast cancer (10–12). Antibodies directed against the extracellular domain of the epidermal growth factor receptor exhibit activity in advanced colorectal cancer (13, 14). Furthermore, antibodies that inhibit T-cell activation by blocking the function of the CTLA-4 co-receptor on T-cells exhibit pre-clinical promise (15) and are undergoing clinical evaluation.

Antibody-dependent cellular cytotoxicity (ADCC) occurs when antibodies bind to antigens on tumor cells and the antibody Fc domains engage Fc receptors on immune effector cells (16). Several families of Fc receptors have been identified, and specific cell populations characteristically express defined Fc receptors (17). For example, polymorphonuclear leukocytes commonly express human FcγRI (CD64), FcγRIII (CD32), and the B (lipid-anchored) isoform of FcγRIII (CD16). In contrast, human natural killer cells express only the A (transmembrane) isoform of CD16. This structure facilitates the recruitment of adaptor proteins and the activation of natural killer cells by antibody engagement of CD16 (18). Many anti-tumor antibodies have been shown to mediate in vitro ADCC. Clynes et al. evaluated the importance of Fc receptor interactions by examining the anti-tumor activities of clinically effective monoclonal antibodies against human tumor xenografts in either wild-type mice or murine FcγRII/III knock-out mice (19). Anti-tumor activity was diminished in the Fcγ receptor knock-out mice and was preserved when only the inhibitory Fcγ receptor isoform was deleted. These data support the concept that Fc receptor interactions underlie anti-tumor efficacy in mice and suggest

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‡ Present address: Centocor, Inc., 145 King of Prussia Rd., Radnor, PA 19087.
§ To whom correspondence should be addressed: Fox Chase Cancer Center, 333 Cottman Ave., Philadelphia, PA 19111. Tel.: 215-728-2480; Fax: 215-728-5339; E-mail: Louis.Weiner@fccc.edu.

The abbreviations used are: ADCC, antibody-dependent cellular cytotoxicity; BsAb, bispecific antibody; ECD, extracellular domain; E/T, effector cell to target cell (ratio); scFv, single-chain Fv; PBS, phosphate-buffered saline; TriBi, trimeric bispecific minibody.
that such interactions may be important for the anti-tumor activity of selected antibodies in the clinic (19). The effector cell populations required for these effects have not been defined but are presumed to include mononuclear phagocytes and/or natural killer cells. Manipulations of Fc domain structure can customize antibody clearance and the interaction of Fc domains with cellular Fc receptors (20–22). Recently, polymorphisms in human FcyRIII have been associated with varying probabilities of clinical response to Rituximab (23). These considerations support efforts to developed antibodies with improved capacity to promote anti-tumor cellular cytotoxicity. One such approach has been to develop bispecific antibodies (BsAbs).

In the nearly 20 years following the pioneering work of David Segal and colleagues (34), numerous BsAbs targeting tumor antigens and effector cell trigger molecules have been developed and shown to redirect cellular cytotoxicity. For example, BsAbs can target tumor antigens and human effector cell trigger molecules on T-cells via CD3/TcR (24, 25) and CD28 (26). BsAbs directed against FcγRII (27) trigger tumor cytotoxicity by neutrophils, monocytes, and macrophages. BsAbs targeting FcyRIII (28–30) promote tumor lysis by macrophages and NK cells, while BsAbs targeting CD44 (31) promote tumor cytotoxicity by NK cells alone. Typically, BsAbs promote in vitro cytotoxicity at low concentrations and have been shown to cause either growth delays or tumor regressions in appropriate animal models (32–35). These properties have led to the testing of several BsAbs in human clinical trials. Several trials have been conducted with chemically linked dimers targeting CD64 and either HER2/neu or the epidermal growth factor receptor, either alone or in conjunction with leukocyte activators such as γ-interferon, granulocyte/macrophage colony-stimulating factor, or granulocyte colony-stimulating factor (36–39) (reviewed in Ref. 40). These approaches thus far have yielded minimal clinical anti-tumor activity. We developed and conducted clinical trials of the 2B1 bispecific monoclonal IgG antibody that targets epitopes on the extracellular domains of HER2/neu and CD16, respectively (41). Therapy with this antibody led to occasional clinical responses and induced the development of host anti-HER2/neu antibodies and T-cell responses directed against both the extracellular and intracellular domains of HER2/neu. This phenomenon of Fc receptor-targeted immunization is best explained by bispecific antibody promotion of tumor lysis, with subsequent antigen presentation (42).

Treatment with 2B1 was associated with dose-limiting toxicities including thrombocytopenia, cytokine-release syndrome, and profound, transient leukopenia induced by the binding of the antibody to multiple Fc receptors through its anti-CD16 and Fc domains. Accordingly, the antibody cross-linked neutrophils and mononuclear phagocytes, leading to cytokine release (41, 43). To address this problem we created recombinant single-chain Fv-based bispecific antibodies that bind to tumor antigens with high affinity (44–46) and to CD16 with a lower affinity. These single-chain Fv dimers contain no Fc domains and do not activate leukocytes in the absence of tumor engagement (47). To investigate the influence of binding affinity for HER2/neu on the capacity to promote tumor cytotoxicity, we subsequently prepared single-chain Fv bispecific dimers with varying binding affinity for HER2/neu and found that higher affinity binding to the tumor antigen corresponds to a greater potential to induce cytotoxicity (48).

Knowing that higher affinity for tumor antigen improves bispecific antibody-promoted cytotoxicity, we sought to determine whether increased valence would have similar effects. To accomplish this, we constructed bispecific minibodies that bind monovalently and divally to HER2/neu and monovalently to CD16. The minibody format was chosen to increase the span of the bispecific molecule so that it approximates more closely the functional binding site of an IgG molecule. We found that these binding formats are more efficient mediators of antibody-promoted cellular cytotoxicity and offer advantages over bispecific single-chain Fv molecules.

**Experimental Procedures**

**Cloning, Expression, and Purification of Minibody Constructs and the CD16 Extracellular Domain (ECD)**

**Bispecific Minibody**—The genes for the anti-HER2/neu and the anti-CD16 scFvs were each cloned into the pBudCE4 dicistronic vector (Invitrogen) adjacent to the Cγ3 gene (kindly provided to us by IDEC) and separated by a short linker (19 and 29 amino acids, respectively). Cloning of the Ig κ-chain signal sequence (Invitrogen) at the 5′-end of each sequence allowed for secretion of the molecule into the culture media. Mutations were made in each of the Cγ3 domains to create the “knobs-into-holes” configuration (49). In the anti-HER2/neu strand the smaller Cγ3 Thr-26 residue was mutated to a larger tyrosine residue by using PCR primers to change the threonine codon, ACC, to the tyrosine codon TAC. In contrast, in the anti-CD16 strand the reverse was performed in that the larger Cγ3 Tyr-67 molecule was converted to a smaller threonine residue by mutating the tyrosine codon, TAC, to the threonine codon, ACC, using PCR primers. Two cysteine residues were added at the 3′-end of each Cγ3 domain to allow for disulfide bond formation, which stabilized the minibody construct. Sequences corresponding to a V5 epitope as well as a His6 tag were cloned at the 3′-end of the anti-HER2/neu binding arm, whereas the anti-CD16 binding arm contains a Myc epitope as well as a His6 tag at its 3′-end. A FLAG epitope was also cloned at the 5′-end of the anti-HER2/neu scFv.

The pBudCE4-bispecific minibody vector was transiently transfected into COS-7 cells and stably transfected into HEK 293 cells. Zeocin was used for the selection of positive HEK 293 cell clones. Cells supernatants were collected, centrifuged, and filtered prior to loading onto a CapturePro column (American Biosciences). Nonspecifically bound proteins were removed from the column by first washing with phosphate buffer containing 10 mM imidazole followed by a second phosphate buffer wash containing 50 mM imidazole. Minibody proteins were then batch eluted using a 500 mM imidazole phosphate buffer fraction. Fractions containing the proteins were combined and dialyzed into PBS. Proteins were subsequently loaded onto an anti-FLAG column (Sigma-Aldrich) at 4 °C for further purification of the bispecific species. Nonspecifically bound proteins were washed away with PBS, and the minibody was batch eluted in 4×1 ml fractions using 0.1 mM glycine, pH 2.8. After visualization on an SDS-PAGE gel, fractions containing the bispecific minibody were combined and dialyzed into PBS. The protein was finally purified over an anti-Myc column (Covance Inc., Princeton, NJ) using the same method employed for purification over the anti-FLAG column. Fractions containing the purified bispecific minibody were combined and dialyzed into PBS. Concentration measurement was determined by a Bio-Rad protein assay (Bio-Rad Laboratories).

**Trimeric Bispecific Minibody (TriBi)**—The trimeric bispecific minibody was cloned similarly to the bispecific minibody. An additional anti-HER2/neu scFv was cloned to the Cγ3 domain of the pre-existing anti-HER2/neu binding arm. The second anti-HER2/neu scFv was separated from the carboxy-terminal end of the Cγ3 domain by a 24-amino acid linker. A FLAG epitope was cloned at the 5′-end, and a V5 epitope was cloned at the 3′-end of the anti-HER2/neu binding arm similarly to the bispecific minibody. However, unlike the parent molecule, the His6 tag was removed from this binding arm to simplify purification of the molecule. The TriBi molecule was expressed and purified as described for the bispecific minibody. However, because of the removal of the histidine tag from one of the binding arms, the anti-Myc column was not required for its purification.

**CD16 ECD**—The CD16A (CD16) extracellular domain was subcloned from the retroviral vector pBMN-ires-EGFp (a kind gift of Dr. Sei-ich Yusa and Dr. Kerry Campbell, Fox Chase Cancer Center, Philadelphia, PA) (50) and into the pSecTag2/Hygro A mammalian expression vector (Invitrogen, Corp.). This plasmid contained an Ig κ-chain leader sequence located 5′ to the CD16 DNA as a means to induce secretion of the expressed CD16 ECD protein into the cell supernatant. The sequences for both a Myc epitope and a His6 tag were located 3′ to the CD16 ECD gene. The pSecTag2/Hygro A-CD16 vector was transiently transfected into COS-7 cells and stably transfected into HEK 293 cells. Hygromycin was used for the selection of positive HEK 293 cells clones.
Cell supernatants were collected, centrifuged, and filtered prior to loading onto a 5-ml His-Trap column (Amersham Biosciences) for protein purification. Purified protein was visualized on an SDS-PAGE gel. Fractions containing the protein were combined and dialyzed into PBS.

**Biacore Analysis**

**Bispecific Minibody**—The binding kinetics of the bispecific minibody binding to the ECDs of both CD16 and HER2/neu were determined by surface plasmon resonance using the Biacore 1000 biosensor system (Biacore Inc., Piscataway, NJ) (51). The bispecific minibody was dialed into 10 mM sodium acetate, pH 5.2 and immobilized on a research grade CM5 sensor chip (Biacore Inc.) by amine coupling (kit supplied by the manufacturer). Unreacted moieties on the chip surface were blocked with ethanolamine. The minibody was applied to a CM5 sensor chip at a flow rate of 5 μl/min for 4 min, resulting in the immobilization of 343 response units on one flow cell (used for the HER2/neu binding experiment) and 180 response units on another flow cell (used for the CD16 binding experiment). Both the HER2/neu and the CD16 ECDs were dialed into PBS and then diluted into concentrations ranging from 10 μM to 320 μM. Binding affinities were analyzed by the continuous flow of the analytes across the appropriate occupied flow cell at a rate of 50 μl/min for 30 min. Each sample also was passed over a control flow cell that had been activated but contained no antigen. The control binding curves were subtracted from the corresponding test curves. As a second control, PBS was passed over the test flow cell. This control curve also was subtracted from the test curves to correct for buffer effects. Following the analysis of each sample, the CM5 sensor chip was regenerated by passing 4 mM MgCl₂, followed by 50 mM triethylenamine at a flow rate of 50 μl/min. Kinetic analysis was performed for each antigen to determine the association (kₐ) and dissociation (k₈) rates as well as the equilibrium constants (Kₐ and K₈).

A two-step binding assay was performed to determine whether the bispecific minibody bound to both of its targets simultaneously. In this experiment the minibody was passed over a CM5 sensor chip containing a high density of the HER2/neu ECD. Once binding was observed, the CD16 ECD was passed over the same (unwashed) flow cell, and a change in response was recorded. As a negative control, an unrelated protein was passed over the HER2/neu-minibody complex, and any change in response was recorded.

**TriBi Minibody**—The TriBi molecule was dialedized into 10 mM sodium acetate, pH 5.2, and coated onto a research grade CM5 sensor chip (Biacore Inc.). Unreacted moieties on the chip surfaces were blocked with ethanalamine. The TriBi was applied to a CM5 sensor chip at a flow rate of 5 μl/min for 4 min, resulting in the immobilization of 334 response units of material. This flow cell was used for both the HER2/neu and the CD16 binding assays, which were performed as described above for the bispecific minibody. To determine that the TriBi minibody possessed bispecific functionality, a two-step binding assay was performed as described above for the bispecific minibody.

**Serum Stability**

Two hundred nanograms of each minibody construct were incubated in both 100% human and 100% mouse serum at 37 °C for up to 72 h. Samples were removed for analysis at 5 min, 6 h, 24 h, and 72 h following the start of incubation. SDS loading dye was added to each sample and frozen until the entire study was completed. Samples were resolved on a 9% polyacrylamide gel under non-reducing conditions and subsequently visualized by anti-histidine Western analysis.

To determine whether minibody constructs remained functional in human serum, both minibody constructs were incubated in 10% serum at 37 °C for 24 h. As a control, a second of serum-exposed samples was frozen immediately in SDS loading dye to represent zero time point. Both minibodies were then tested for their capability to retain functional binding using surface plasmon resonance on the Biacore 1000 instrument. The HER2/neu ECD was coated onto the surface of a research grade CM5 sensor chip at a high density. Each of the minibody constructs was passed over the flow cell containing the immobilized HER2/neu ECD at a flow rate of 15 μl/min, and a change in response was monitored. Ten percent serum alone was also passed over the chip as a negative control.

**Cytotoxicity Assay**

These studies were performed as described previously (29). Briefly, human CD16-transduced NK-92 cells (a kind gift of Dr. Kerry Campbell, Fox Chase Cancer Center, Philadelphia, PA and of the ZelleRx Corporation (Chicago, IL)) served as the effector cells (E) for this assay. The cells were cultured in α-minimal Eagle’s medium, 12.5% fetal bovine serum, 12.5% horse serum, 2 mM l-glutamine, 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, and 0.02 mM folate acid. Cells were restimulated with fresh medium containing human interleukin-2 1 day prior to the assay. SK-OV-3 targets (T) were labeled with Na¹⁰⁵C²⁰⁴ (100 μCi/10⁶ targets; PerkinElmer Life Sciences) for 1 h at 37 °C in supplemented RPMI 1640. The ¹⁰⁵C²⁰⁴-labeled SK-OV-3 target cells were washed twice and resuspended at the desired concentration. Ten thousand cells were added to individual wells of 96-well flat-bottomed plates (Costar, Cambridge, MA) containing hNK-92 cells and/or antibody in supplemented RPMI 1640. Effector cells were added to yield E/T ratios of 25:1, 1:1, and 5:1 in the presence or absence of various concentrations of antibody. Each well contained a total volume of 200 μl, and all assays were performed in triplicate. The plates were centrifuged at 300 × g for 3 min, incubated for 4 h in a 5% (v/v) CO₂ incubator at 37 °C, and then centrifuged again at 300 × g for 3 min. One hundred microliters of supernatant were removed from each well for counting on a Packard Instruments Cobra Quantum, Series 5002 (PerkinElmer Life Sciences). Cytotoxicity was estimated by measuring the quantity of label released into culture supernatants using the formula shown in Equation 1,

\[
\text{% Lysis} = 100 \times \frac{\text{Experimental release (cpm)} - \text{Spontaneous release (cpm)}}{\text{Total counts (cpm)/2} - \text{Spontaneous release (cpm)}}
\]

where the experimental release was defined as counts per minute (cpm) obtained by target cells in the presence of effector cells and/or antibody and the spontaneous release was defined as cpm released by target cells alone. Antibodies tested in this assay included the bispecific minibody, the TriBi minibody, ML3.9-Y3, a bispecific (scFv)₂ that targets HER2/neu, and CD16 using the same scFv sequences used in the minibody constructs, trastuzumab, and 2B1. Antibody concentrations tested ranged from 0.005 to 500 μg/ml for each antibody.

**RESULTS**

**Cloning, Expression, and Purification of Minibody Constructs**—Both the bispecific and the trimeric bispecific minibodies were cloned into the pBudCE4 mammalian vector. In each case the scFv genes specific for either the HER2/neu or the CD16 antigens were subcloned into the pBudCE4 mammalian vector. In vector the scFv genes specific for either the HER2/neu or the CD16 antigens were subcloned into the pBudCE4 mammalian vector. In vector the scFv genes specific for either the HER2/neu or the CD16 antigens were subcloned into the pBudCE4 mammalian vector.
Minibody Construct Binding to the HER2/neu and CD16 Antigens—To test whether the scFv portions of the minibody would bind to their respective targets in the minibody format, surface plasmon resonance was performed. The bispecific minibody was coated onto a CM5 Research Grade chip (Biacore Inc.) at low density through an amine-coupling reaction. The HER2/neu ECD or the CD16 ECD was then passed over the appropriate flow cell at a rate of 50 μl/min at various concentrations ranging from 10 to 320 nM for both analytes.

The bispecific minibody binds to the HER2/neu ECD with an affinity ($K_D$) of $7.6 \times 10^{-8}$ M ($k_a = 3.3 \times 10^4; k_d = 2.5 \times 10^{-4}$) (Table IA) and to the CD16 ECD with an affinity ($K_D$) of $6.25 \times 10^{-8}$ ($k_a = 2.6 \times 10^5; k_d = 1.6 \times 10^{-3}$) (Table IB). Table IC describes the results from a two-step binding assay performed to demonstrate that the molecule had bispecific functionality. In this assay, the bispecific minibody was allowed first to bind to the HER2/neu ECD on the sensor chip. The CD16 ECD was then injected immediately and allowed to bind to the HER2/neu-minibody complex, which resulted in a positive change in response units. The bispecific minibody was able to bind to both targets simultaneously, demonstrating its bispecific functionality.

Similar experiments were performed with the TriBi molecule in that the TriBi was first coated onto a CM5 Research Grade chip (Biacore Inc.) at a low density followed by the addition of the HER2/neu ECD and the CD16 ECD analytes, respectively, at concentrations ranging from 10 to 320 nM. Kinetic analysis of the TriBi minibody showed that this construct binds monovalently to the HER2/neu ECD with a $K_D$ of $1.8 \times 10^{-8}$ M ($k_a = 1.2 \times 10^5; k_d = 2.3 \times 10^{-4}$) (Table IA). The affinity of the TriBi molecule for the CD16 ECD was also measured and determined to bind with a $1.2 \times 10^{-7}$ M $K_D$ ($k_a = 1.4 \times 10^5; k_d = 1.7 \times 10^{-3}$) (Table IB). Further analysis indicated that this molecule could also bind to both targets simultaneously, thus demonstrating bispecific functionality (Table IC).

Serum Stability—Testing the stability of engineered antibody fragments in human as well as in mouse serum is critical before proceeding to in vivo assays. In these studies the minibody constructs were incubated in both 100% human and 100% mouse serum at 37 °C for up to 72 h. Samples were obtained at 5 min, 6 h, 24 h, and 72 h following the start of incubation, resolved on a 9% polyacrylamide gel under non-reducing conditions, and visualized by anti-histidine Western analysis. Although some nonspecific binding to serum proteins was observed (upper band in Fig. 3B), no minibody breakdown products were present. These results indicate that both minibody molecules are stable up to 72 h under physiological conditions (Fig. 3, A–D).

The bispecific minibody and the TriBi minibody retained binding functionality in human serum. In this experiment, each construct was first incubated in 10% human serum at 37 °C for 24 h. The samples were then tested for their ability to bind to the HER2/neu ECD, which was immobilized onto a CM5 sensor chip and analyzed by surface plasmon resonance (Biacore Inc.). Both minibodies bound to the HER2/neu ECD after incubation in 10% human serum at 37 °C (Fig. 4, A and B).
**Cytotoxicity**—Standard 4-h $^{51}$Cr release assays were performed to determine whether the anti-HER2/neu-anti-CD16 bispecific minibody format could induce the cytotoxicity of tumor cells that overexpress HER2/neu by CD16-expressing mononuclear cells. Human ovarian carcinoma cells of the SK-OV-3 line, which overexpress HER2/neu, were used as the target cells, and the human NK cell line hNK-92, transduced with human CD16, was used as the source of effector cells. Assays were conducted at E/T ratios of 5:1, 10:1, and 25:1 at antibody concentrations ranging from 0.005 to 500 nM. Each sample was plated in triplicate, and one control set of wells contained both effector and target cells but no antibody. 2B1, trastuzumab, and the bispecific scFv molecule ML3.9-Y3 were also tested in each cytotoxicity experiment. The ML3.9-Y3 (scFv), molecule is similar to the minibody constructs in that it possesses the identical anti-HER2/neu and anti-CD16 scFv targeting moieties; however, the scFv moieties are covalently fused in a smaller format joined by a 15-amino acid linker. At a 5:1 E/T ratio, a 50 nM concentration of the TriBi molecule promotes cytotoxicity equivalent to 2B1 and trastuzumab. This concentration is 10-fold higher than the concentration at which both 2B1 and trastuzumab achieve maximal lysis (Fig. 5). The concentration of the TriBi construct required to induce half-maximal lysis is lower than that of the bispecific minibody. Thus, the TriBi, which binds divalently to HER2/neu, promotes more cytotoxicity than the corresponding bispecific minibody, which binds monovalently. Both molecules induce cytotoxicity superior to that of the single-chain Fv dimer, which contains essentially similar affinities and specificities for the identical HER2/neu and CD16 epitopes (Fig. 5).

**DISCUSSION**

The results presented here indicate that divalent binding to a tumor antigen is preferable to monovalent binding for the promotion of ADCC. Previously, we had demonstrated that affinity for tumor antigen regulates the capacity of bispecific scFv dimers targeting HER2/neu and CD16 to promote cytotoxicity (48). However, cytotoxicity potentiation did not approach that seen with either conventional or bispecific IgG antibody formats. Accordingly, we sought to determine whether placing the scFv components of the bispecific dimer into a minibody format would enhance cytotoxicity, presumably by facilitating bispecific binding through the larger size and flexibility of the minibody binding format. We demonstrate a feasible approach to creating bispecific minibodies using a trimeric, bispecific minibody format possessing many of the advantages of a native IgG with respect to flexibility and the capacity to bridge its target antigens while retaining a sufficiently small size to promote highly selective in vivo tumor retention (52).

A number of groups have demonstrated the ability to generate monospecific, bispecific, and trispecific recombinant antibodies containing a variety of dimerization motifs. scFv dimers were first reported in 1993 and were shown to mediate improved selective tumor targeting as compared with monomeric scFv and IgG, respectively (55). Bispecific recombinant scFv dimers have been shown to mediate retargeted cytotoxicity as well (47, 48, 54). Dimeric minibodies have been constructed employing either $\text{C}_1\text{N}_2$, $\text{C}_1\text{C}_3$, or $\text{C}_1\text{C}_6$ dimerization motifs; such molecules have been monospecific (49, 52, 55) or bispecific (56). Trimeric molecules have been constructed employing diabodies wherein short linkers between $\text{V}_\text{H}$ and $\text{V}_\text{L}$ chains require that the respective chains fold heterologously; e.g. $\text{V}_\text{H}$ associates with $\text{V}_\text{L}^2$, and $\text{V}_\text{L}^2$ associates with $\text{V}_\text{L}^1$ (57–59). When the linker is sufficiently short, trimers form (e.g. tribodies), and

**TABLE I**  
Minibody binding assessed by surface plasmon resonance

| Antibody type        | $k_\alpha$ | $k_\beta$ | $K_d$  |
|----------------------|------------|------------|--------|
|                      | $M^{-1}s^{-1}$ | $s^{-1}$ |        |
| Bispecific minibody  | $3.3 \times 10^4$ | $2.5 \times 10^{-4}$ | $7.6 \times 10^{-9}$ |
| TriBi minibody       | $1.2 \times 10^4$ | $2.3 \times 10^{-4}$ | $1.8 \times 10^{-8}$ |

**C. Dual Binding to HER2/neu and CD16**

| Second protein | Bispecific minibody | TriBi minibody |
|----------------|---------------------|----------------|
| CD16           | 43                  | 14.3           |
| Negative Control | -11.4            | -2.0           |

* Additional change in response units (RU) when the indicated protein flows over a HER2/neu ECD-coated flow cell previously exposed to the indicated antibody format. A positive value indicates that antibody bound to the HER2/neu ECD simultaneously binds to the indicated protein.
these molecules can contain multiple binding specificities (58, 60).

The novelty of the work described here rests in the construction of a trimeric, bispecific minibody and its dimeric, bispecific counterpart to permit a comparison of the effects of valence for tumor antigen on the capacity of the respective constructs to promote ADCC. As shown in Fig. 5, the trimeric, bispecific construct containing two binding sites for HER2\text/neu exhibited a higher degree of cytotoxicity at all E/T ratios tested and at all but the highest (500 nM) antibody concentrations tested. These findings show that divalent binding to a tumor antigen improves the efficiency of antibody-promoted cytotoxicity. This result is not unexpected, because divalent binding prolongs cell surface retention and thus increases the opportunity for the anti-CD16 antibody domain to engage effector cells and promote cytotoxicity. This could be particularly important at low E/T ratios and/or low antibody concentrations.

We observed that at a 500 nM antibody concentration the bispecific minibody construct was significantly more potent than the bispecific scFv dimer, which contains the identical scFv binding domains for HER2\text/neu and CD16, indicating the value of converting the binding sites into this novel minibody format. In addition, the trimeric bispecific minibody was also significantly more potent than the bispecific scFv dimer in the cytotoxicity assay, and a 100-fold lower concentration of antibody achieved equivalent target cell lysis in comparison with the bispecific minibody molecule. Although other trimeric single chain fragments have been described in the literature previously (61, 62), the trimeric minibody design provides the benefits of a more “IgG-like” antibody without the Fc region to avoid systemic leukocyte activation and a molecule with greater “wingspan” and flexibility than either the single chain dimers or single chain trimers. Therefore, the primary objective behind designing these modifications was to create a molecule with improved ADCC capabilities over its single-chain counterparts. Our findings demonstrate that although increas-
ing the wingspan of the antibody did improve its potency, a combination of divergent tumor binding and greater flexibility was required to induce more effective cytotoxicity.

Interestingly, neither minibody format was as efficient as either trastuzumab or the 2B1 bispecific IgG in promoting cytotoxicity. Both trastuzumab and 2B1 target distinct epitopes on the HER2/new ECD, and it is possible that the nature of the target epitope contributes to the susceptibility to lysis. Perhaps more importantly, the Fc receptor binding domains of each of these antibodies differ. Trastuzumab contains a human IgG1 Fc domain, whereas 2B1 exerts the bulk of its in vitro cytotoxicity through its binding to the CD16 epitope recognized by the 3G8 monoclonal antibody (29). The anti-CD16 scFv used in the minibody constructs was selected because of its lower affinity for its target as a means to minimize unwanted systemic leukocyte activation by a bispecific antibody in the absence of tumor cell engagement (47, 48). Hence, these epitope differences may account for some of the variations observed in the cytotoxicity promotion potential of the tested antibodies. It also is conceivable that the IgG format remains superior to other antibody structures with respect to cytotoxicity promotion because of differences in binding site flexibility or the capacity to bridge effector cell and target cell ligands. However, other investigators have identified smaller scFv-based bispecific formats with considerable cytotoxic potency (54). Previous studies have examined the cytotoxic properties of bispecific antibodies as compared with conventionally structured IgG molecules; however, although these studies have compared antibodies that bind to their tumor targets monovalently (bispecifics) and divalent (IgG), the binding of these molecules to Fc receptors have had different structural bases, making it impossible to directly compare monovalent and divalent tumor antigen binding effects on antibody-promoted cytotoxicity. Additionally, the question of what level of ADCC is required to cause significant in vivo tumor regression still remains unanswered. It is possible that the level of ADCC potentiated by the TriBi minibody, for example, will be high enough to cause an attenuation of tumor growth. These studies are currently underway.

We prepared the bispecific minibody and TriBi constructs employing the knobs-into-holes CH3 heterodimerization domain, previously described by Ridgway et al. (49). The resulting molecules had properties similar to those of the corresponding molecules prepared without forced heterodimerization. Such antibodies required purification by sequential affinity chromatography procedures that reduced the yields of the final purified product. However, purification was more straightforward and more efficient using the heterodimerization approach (data not shown). It was encouraging to observe that the bispecific minibody and TriBi molecules were stable in human and mouse sera for at least 14 days (data not shown). It was encouraging to observe that the bispecific minibody and TriBi molecules were stable in human and mouse sera for at least 14 days (data not shown).
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