Development of Humanized Bispecific Antibodies Reactive with Cytotoxic Lymphocytes and Tumor Cells Overexpressing the *HER2* Protooncogene

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Summary

The *HER2* protooncogene encodes a 185-kD transmembrane phosphoglycoprotein, human epidermal growth factor receptor 2 ($p185^{HER2}$), whose amplified expression on the cell surface can lead to malignant transformation. Overexpression of *HER2*/$p185^{HER2}$ is strongly correlated with progression of human ovarian and breast carcinomas. Recent studies have shown that human T cells can be targeted with bispecific antibody to react against human tumor cells in vitro. We have developed a bispecific $F(ab')_2$ antibody molecule consisting of a humanized arm with a specificity to $p185^{HER2}$ linked to another arm derived from a murine anti-CD3 monoclonal antibody that we have cloned from UCHT1 hybridoma. The antigen-binding loops for the anti-CD3 were installed in the context of human variable region framework residues, thus forming a fully humanized $BsF(ab')_2$ fragment. Additional variants were produced by replacement of amino acid residues located in light chain complementarity determining region 2 and heavy chain framework region 3 of the humanized anti-CD3 arm. Flow cytometry analysis showed that the bispecific $F(ab')_2$ molecules can bind specifically to cells overexpressing $p185^{HER2}$ and to normal human peripheral blood mononuclear cells bearing the CD3 surface marker. In additional experiments, the presence of bispecific $F(ab')_2$ caused up to fourfold enhancement in the cytotoxic activities of human T cells against tumor cells overexpressing $p185^{HER2}$ as determined by a ^{51}Cr release assay. These bispecific molecules have a potential use as therapeutic agents for the treatment of cancer.

Recent studies have revealed an association between overexpression of the *HER2* protooncogene and the progression of breast and ovarian carcinomas accompanied by worsened clinical outcome (1–3). *HER2* encodes a transmembrane phosphoglycoprotein receptor tyrosine kinase with an approximate molecular weight of 185,000 ($p185^{HER2}$) whose amplified expression can lead to malignant transformation as determined in soft agar assays and in nude mice models (4, 5). Thus, *HER2* may play a crucial role in the tumorigenesis of breast and ovarian carcinomas in humans (2). Of relevance, cells overexpressing *HER2*/$p185^{HER2}$ exhibit more resistance to cytotoxic effects of monocytes and TNF-α, a cytokine that has direct antitumor activities and is thought to mediate immune cell killing of tumor cells (5).

Bispecific mAbs (BsmAbs) with dual specificities for tumor-associated antigens on tumor cells and for surface markers on immune effector cells have been described (6, 7). These BsmAbs have been shown to be effective in directing and triggering effector cells to kill tumor cell targets (8). One approach to produce BsmAb involves the fusion of two mAb-producing hybridomas to form quadromas that secrete BsmAb in addition to undesirable chain combinations including parental mAbs. Another approach utilizes directed chemical coupling of Fab' fragments from two different mAbs to assemble a BsmAb with the desired specificities (9). Limitations associated with such approaches include the ability of rodent-derived BsmAb to elicit immune response in humans. To this end, genetic engineering techniques have been applied to production of less immunogenic "humanized" antibodies (10, 11). Recently, we have described the humanization of murine mAb.4D5 (mumAb4D5), which is directed against the extracellular domain (ECD) of $p185^{HER2}$ The

[1] *Abbreviations used in this paper:* BsmAb, bispecific mAb; FR, framework region; hu, humanized; $p185^{HER2}$, human epidermal growth factor receptor 2.

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humanized (hu) antibody, humAb4D5-8, consists of the antigen-binding hypervariable regions from the murine parent mAb together with human variable region framework residues and constant domains (12).

In this report, we describe the production of humanized BsF(ab')$_2$ with specificities toward the extracellular domain of p185^{HER2}, and the human T cell surface marker CD3. Thus, one arm is humAb4D5-8 and the other a humanized version of murine anti-CD3 mAb, UCHT1. Our approach involved separate *Escherichia coli* secretion of each Fab' followed by directed chemical coupling reaction in vitro to form the BsF(ab')$_2$ fragment. Data are presented demonstrating the biological properties of these BsF(ab')$_2$ molecules, including the specific binding to cells overexpressing p185^{HER2} and to normal human T cells; and their ability to trigger the lytic activity of human CTL against breast tumor targets.

Materials and Methods

Cloning of Anti-CD3 Variable Region Genes. The mumAb anti-CD3–producing hybridoma UCHTI (13) was used for extraction of mRNA (14). The genes encoding mumAb anti-CD3 variable domains were isolated by PCR amplification of mRNA as described (15). NH_2-terminal sequencing of mumAb anti-CD3 light and heavy chains was used to design the sense strand PCR primers, whereas the antisense PCR primers were based upon consensus sequences of murine FR residues (16) incorporating unique restriction sites for directional cloning shown by underlining and listed after the sequences: V_L sense, 5' TTTAACGCGTACGCTGACATCCAGATGACCCAGACCACCTCCTCCCTGTCTGCCTCCCTGGGCGAY3', *Mlu*I; V_L antisense, 5' TTTGCATGCGTCTTTGGGGTAGAAGTTGTTCAAGAAGCA 3', *Sph*I; V_H sense, 5' AACGCGTACGCTGARGTSCARCTSCARCARTC 3', *Mlu*I and V_H antisense 5' GGCAGAGATCCAGGGGCCCGTGGATAGACAGATGG 3', *Apa*I; where Y = T or C, R = A or G, and S = C. For each variable domain, the products from two independent PCR reactions were cloned into the pUC119-based phagemid pAK2 (12), and a total of at least five clones was sequenced by the dideoxy method (17).

Molecular Modeling and Construction of Humanized mAb Anti-CD3 Genes. Humanization of mumAb anti-CD3 by installing CDR residues from this murine antibody into the context of consensus human FR sequences was performed as previously described for mumAb4D5-8 (12). Briefly, mumAb anti-CD3 was humanized by judicious recruitment of corresponding CDR residues and a few FR residues into the humAb4D5-8 molecule. Differences between mumAb anti-CD3 and the human consensus FR residues (see Fig. 1) were individually modeled to investigate their possible influence on CDR conformation and/or binding to CD3. Genes encoding humAb anti-CD3 variant 1 V_L and V_H domains were assembled by gene conversion mutagenesis of corresponding humAb4D5 gene segments cloned in pUC119 (12) using 246-mer and 283-mer preassembled oligonucleotides, respectively. Briefly, sets of four contiguous oligonucleotides were designed to create humAb anti-CD3 V_H and V_L utilizing codons commonly found in highly expressed *E. coli* genes (see Fig. 1). These oligonucleotides are 54–85 residues in length, contain 7–17 mismatches to the humAb4D5 templates, and are constrained to have eight or nine perfectly matched residues at each end to promote efficient annealing and ligation of adjacent oligonucleotides. The sets of oligonucleotides were phosphorylated, annealed to corresponding templates, and ligated. Full-length oligomers were isolated after electrophoresis on a 6% acrylamide sequencing gel and then used for an efficient mutagenesis procedure (12). Clones precisely encoding mumAb anti-CD3 V_H and V_L (Fig. 1) were identified by nucleotide sequencing (17).

Additional humAb anti-CD3 variants were designed in which one or more mumAb anti-CD3 residues were replaced by their human counterparts in order to test their role in antigen binding, namely V_H K73D and/or V_L R53S (a single letter code for the original amino acid followed by the residue number according to Kabbat et al. [16] and then the amino acid replacement). The murine V_H residue, K73, is located in a loop that is close to heavy chain CDRs H1 and H2 (Fig. 2), and might be involved in antigen binding. In contrast, our humAbs usually contain an aspartate at this position as they are derived from a consensus sequence of V_H group III, which is the most abundant human group in the compilation of Kabat et al. (16). V_L residue 53 is an arginine in mumAb-antiCD3 that is located towards the COOH terminus of CDR L2 and may be able to reach up and assist in antigen binding while also interacting with the phenyl ring of V_L Y50 (see Fig. 2). In our humAbs, this residue is normally a serine derived from a consensus sequence of V_L κ subgroup I, which is the most abundant human light chain subgroup (16). These additional variants were generated by an efficient site-directed mutagenesis method (18) using the oligonucleotides:

V_L R53S
5' CTATACCTCC*A*GCC*T*CGAGTCTAGGAG 3'
XhoI

V_H K73D
5' AAGCGTAGATGACTCCAAAAACACAGCCTACCTGCAAAT
*G*AA*T*T*C*TCTGCGTGCTG 3'
EcoRI

where an asterisk indicates a mismatch and unique restriction sites introduced are underlined.

Fab' Fragment Expression for humAb4D5-8 and Anti-CD3 mAb Variants. We previously described the vector, pAK19, for the cosecretion of humAb4D5 light chain and heavy chain Fd' fragments from *E. coli* (19), which is available upon request to the authors. Briefly, the Fab' expression unit is dicistronic with both chains under the transcriptional control of the *pho*A promoter (20), ends with the bacteriophage λ t_0 transcriptional terminator (21) and is cloned between the EcoRI and HindIII site of pBR322 (22). The humanized V_L and V_H domains (12) are precisely fused on their 5' ends to a gene segment encoding the heat-stable enterotoxin II signal sequence (23) and on their 3' side to human κ_1 (C_L) (24) and IgG1 (C_H1) (25), followed by the hinge sequence CysAlaAla (19) constant domains, respectively. Chimeric and humanized versions of mumAb anti-CD3 Fab' were constructed by precisely replacing gene segments encoding humAb4D5-8 V_L and V_H in pAK19 with appropriate genes encoding mAb anti-CD3 V_L and V_H variants by subcloning (26) and site-directed mutagenesis as described (12). Fab' fragments were expressed in a phage-resistant derivative of *E. coli* strain RV308 (27) at high cell density in the fermentor as previously described (19).

Construction of Bispecific F(ab')$_2$ Fragments. Intact and functionally active humAb45D5-8 Fab' has been recovered from *E. coli* fermentation pastes with the unpaired hinge cysteine present mainly (75–90%) in the labile-free thiol form (Fab'-SH) by affinity purification using Streptococcal protein G at pH ~5 in the presence of 10 mM EDTA (19). The anti-CD3 mAb Fab'–SH was recovered by similar procedures and reacted with 5,5'-dithiobis (2-

nitrobenzoic acid) (DTNB) (28) to form the thionitrobenzoate derivative (Fab'-TNB). The construction of bispecific (Bs) $F(ab')_2$ fragments was completed by directed chemical coupling (29) of Fab'-TNB derivative of the anti-CD3 mAb with humAb4D5-8 Fab'-SH. Equimolar quantities of Fab'-TNB (by TNB content) and Fab'-SH (by SH content) were coupled at a combined concentration of ≥0.25 mg/ml in the presence of 100 mM Tris-HCl, pH 7.5, and 10 mM EDTA for 1 h at 37°C. The resulting $BsF(ab')_2$ fragments were isolated from the coupling reaction by S100-HR gel filtration (Pharmacia Fine Chemicals, Piscataway, NJ) in the presence of PBS. The $BsF(ab')_2$ samples were passed through a sterile 0.2-μm filter and stored either at 4°C or flash frozen in liquid nitrogen and stored at −70°C until used.

Cell Lines. Breast tumor cell lines SK-BR-3 and MDA-MB-175 were purchased from the American Type Culture Collection (Rockville, MD). NR6/10 cells (NIH 3T3 fibroblasts overexpressing $p185^{HER2}$) were obtained from Dr. D. Slamon, University of California (Los Angeles, CA). With the exception of MDA-MB-175, these cell lines overexpress *HER2*/$p185^{HER2}$ as reported (3). The cells were grown in an equal mixture of DME and F12 Ham (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated (30 min, 56°C) FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml Streptomycin (Gibco Laboratories) (complete medium).

Human Cytotoxic Lymphocytes. Blood of normal volunteers was drawn into heparinized syringes, mixed with an equal volume of PBS layered onto Ficoll/Hypaque gradient (specific gravity 1.077) and centrifuged for 45 min at 400 *g*. The separated band of PBMC was aspirated, washed three times in ice cold PBS, and resuspended in complete medium. PBMC were depleted of monocytes by adherence to plastic for 60 min in 100 × 60-mm plates (Costar Corp., Cambridge, MA) at 37°C–5% CO_2. Nonadherent PBMC were activated by incubation in the presence of IL-2 for 24 h and were used as effector CTL against ^{51}Cr-labeled tumor targets in a 4-h ^{51}Cr release cytotoxicity assay (30). In some experiments, effector CTLs were tested against targets prepared by PHA blastogenic stimulation of PBMC obtained from the same donor of effector cells as detailed previously (30).

Cytotoxicity Assay. SK-BR-3, or NR6/10 cells (3×10^6/ml), were labeled with 150 μCi of $Na_2{}^{51}CrO_4$ (Amersham Corp., Arlington Heights, IL) for 60 min, washed, adjusted to 10^4 cells/50 μl of complete medium, and dispensed into round-bottomed microtiter plates containing quadruplicates of various numbers of effector CTLs in 100 μl of complete medium. Various concentrations of Bs $F(ab')_2$ fragments alone or mixed with $p185^{HER2}$ ECD were then added in 50-μl volumes (final volume per well = 200 μl) and the plates were incubated at 37°C–5% CO_2. After 4 h, the supernatants were harvested (Skatron Inc., Sterling, VA), and their radioactivity was determined using a gamma counter (Micromedic Systems, Inc., Horsham, PA). Percent cytotoxicity was calculated as follows: percent cytotoxicity = 100× (A − B)/(C − B); where *A* represents the mean counts per minute (cpm) in test supernatants, *B* represents the mean cpm in supernatants of targets alone (spontaneous ^{51}Cr release), and *C* represents the mean cpm in supernatants of targets lysed with 1% SDS (maximum ^{51}Cr release).

Flow Cytometric Analysis of $BsF(ab')_2$ Binding. Aliquots of 10^6 cells were mixed in either PBS + 1% FCS (PBS + 1%) or PBS + 1% containing chimeric or humanized $BsF(ab')_2$ (10 μg/ml). The cells were incubated on ice for 45 min, washed twice in PBS + 1%, and stained with fluorescein-labeled goat anti-human Fab (Tago Inc., Burlingame, CA) for 45 min. In experiments involving the blocking of $BsF(ab')_2$ binding, cells were treated with $BsF(ab')_2$ antibody in the absence or presence of soluble $p185^{HER2}$ extracellular domain preparation of the $p185^{HER2}$, or rCD4 receptor as a negative control (50 μg/ml) before addition of the fluorescein-labeled reagent. The stained cells were washed four times in ice-cold PBS + 1% and analyzed using a FACS® (Becton Dickinson & Co., Mountain View, CA). 10^5 cells were acquired by list mode, and measurements were performed on a single-cell basis and were displayed as frequency distribution histograms. Dead cells and debris were gated out of the analysis on the basis of forward light scatter.

Results

Humanization of mumAb Anti-CD3 V_L and V_H. The gene segments encoding mumAb anti-CD3 V_L and V_H were first cloned by PCR from the corresponding hybridoma, UCHTI, and sequenced (Fig. 1). Next, the deduced variable domain amino acid sequences and molecular modeling were used to design a humanized variant of mumAb anti-CD3 (v1) (Fig. 2) as previously described for mumAb4D5 (12). Corresponding genes for humanized anti-CD3 v1 were created by gene conversion mutagenesis (12) starting from humAb4D5 genes and using long preassembled oligonucleotides (Fig. 1). As detailed in Materials and Methods, further humanized anti-CD3 variants were created by replacement of two additional residues from mumAb anti-CD3 with their human counterparts to investigate their role in antigen binding. Thus, humanized anti-CD3 v2 and v3 incorporate the replacements V_H K73D and V_L R53S, respectively, whereas v4 includes both of these changes.

Preparation of $BsF(ab')_2$ Fragments. We have previously described the secretion of functional humAb4D5 Fab' fragments from *E. coli* at titers of 1–2 g/liter as judged by antigen-binding ELISA after affinity purification on Staphylococcal protein A (19). Chimeric and humanized versions of anti-CD3 were expressed in the same vector (pAK19) at titers of up to 400 mg/liter as judged by total Ig ELISA. Fab' fragments were recovered from *E. coli* cell pastes with the hinge cysteine present mainly (75–90%) as the free thiol (Fab'-SH). This was achieved by affinity purification of Fab'-SH on Streptococcal protein G at pH 5 to maintain the thiol in the less reactive protonated form and in the presence of EDTA to chelate metal ions capable of catalyzing disulfide bond formation. Bs $F(ab')_2$ fragments were then constructed by directed chemical coupling in vitro of humAb4D5 Fab' and anti-CD3 mAb Fab' using the procedure of Brennan et al. (29). One arm of the $BsF(ab')_2$ was always the most potent humanized anti-$p185^{HER2}$ variant previously identified (humAb4D5-8), which binds $p185^{HER2}$ ECD threefold more tightly than the murine parent Ab (12). The other arm was either a chimeric or humanized variant of the anti-CD3 mAb. Henceforth in this text the term chimeric $BsF(ab')_2$ refers to a molecule in which one arm is the humanized anti-$p185^{HER2}$ and the other arm is the chimeric anti-CD3; and the terms $BsF(ab')_2$ v1, v2, v3, and v4 describe a molecule in which one arm is humanized anti-$p185^{HER2}$ and the other arm is humanized anti-CD3 variant 1, 2, 3, and 4, respectively (Fig. 2).

Specific Binding of $BsF(ab')_2$ Fragments to Cells. Binding of $BsF(ab')_2$ fragments to NR6/10 cells overexpressing HER2/

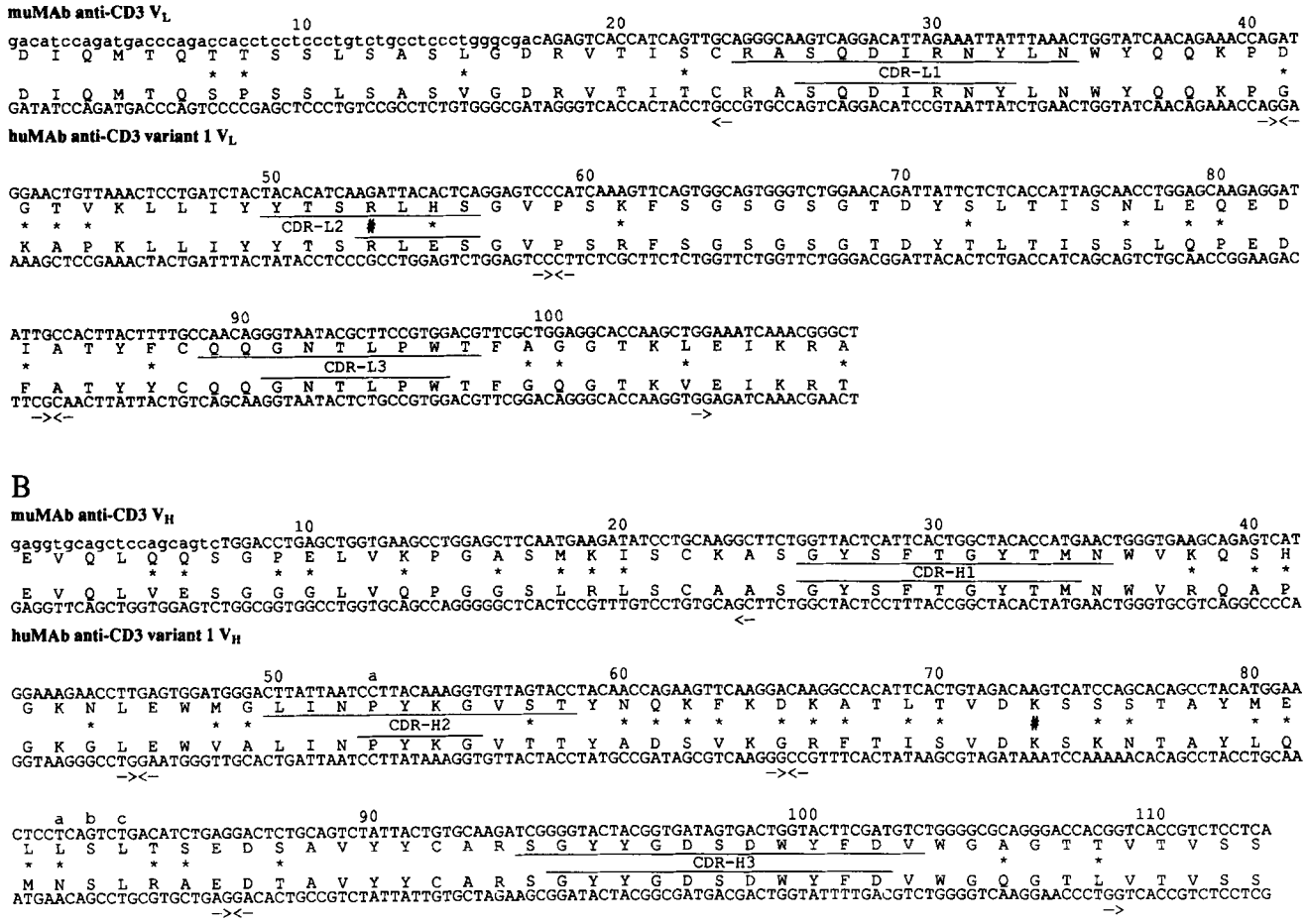

Figure 1. Amino acid and nucleotide sequences of mumAb anti-CD3 and humAb anti-CD3 variant 1 V_L (*A*) and V_H (*B*). Amino acids are numbered using the scheme of Kabat et al. (16) to accommodate differences in V domain lengths. For example, the residues between V_H positions 82 and 83 are designated 82a, 82b, and 82c, respectively. The 5′ end of mumAb anti-CD3 V_L and V_H nucleotide sequences are derived from the corresponding PCR primers and are identified by lower case (see Materials and Methods). Amino acid sequence differences between mumAb and humAb anti-CD3 are shown by asterisks, and residues that were replaced in generating additional humanized variants are indicated by pound signs (see text). The CDR residues according to sequence (16) and structural (38) definitions are shown by overlining and underlining of the CDR label, respectively. Genes encoding humAb anti-CD3 V_L and V_H variant 1 were created by gene conversion mutagenesis (12) of corresponding mumAb4D5 genes using sets of four contiguous oligonucleotides whose 5′ and 3′ ends are shown by arrows below the sequences (see Materials and Methods).

p185^{HER2} was investigated by flow cytometric analysis (Fig. 3). Chimeric BsF(ab′)$_2$ significantly bound to NR6/10 cells as shown by a significant increase in the fluorescence intensity compared with background level (*A* and *B*). The presence of p185^{HER2} ECD (50 μg/ml) in the binding assay reduced the binding of chimeric BsF(ab′)$_2$ almost to background level (*C*), whereas addition of an irrelevant soluble receptor (rCD4) at a similar concentration did not interfere with the binding (*D*). These results demonstrate the specificity of chimeric BsF(ab′)$_2$ binding to cells overexpressing p185^{HER2}. Similar results were obtained using BsF(ab′)$_2$ v1–v4.

Additional FACS® experiments were performed to quantify the binding of BsF(ab′)$_2$ fragments to human CTLs and to breast tumor SK-BR-3 cells. All Bs F(ab′)$_2$ fragments bound to SK-BR-3 cells with equal efficiency as anticipated since the anti-p185^{HER2} arm is identical in these molecules (Fig. 4, *left*). In contrast, there were significant differences in the binding efficiency of these molecules to human CTLs. Chimeric BsF(ab′)$_2$ antibody was most effective in binding (Fig. 4, *right*, *B*) followed by BsF(ab′)$_2$ v1 (*C*), v3 (*E*), and v2 (*D*), as reflected by different peaks of fluorescence intensities; BsF(ab′)$_2$ v4 antibody was least effective in its binding to cytotoxic lymphocytes (*F*). Thus, the manipulation of the anti-CD3 arm of the BsF(ab′)$_2$ fragments profoundly altered its binding to lymphocytes.

Targeting of Tumor Cell Killing by BsF(ab′)$_2$ Fragments. The killing of cells overexpressing p185^{HER2} (NR6/10) or breast tumor cells SK-BR-3 by activated human cytotoxic lymphocytes was examined in the presence of various doses of BsF(ab′)$_2$, and preliminary results indicated that as little as 10 ng/ml of chimeric BsF(ab′)$_2$ or BsF(ab′)$_2$ v1 was sufficient to cause maximal enhancement in the cytotoxic activity of CTLs. This dose (10 ng/ml) was used to compare the ability

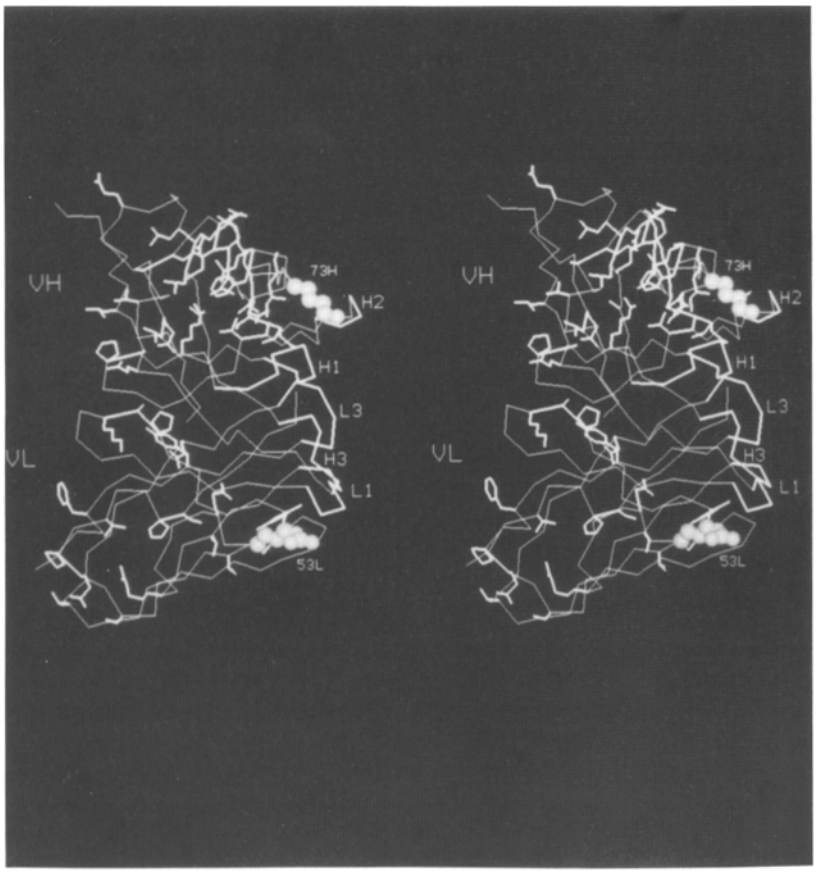

Figure 2. Stereoview of humanized anti-CD3 Variant 1 V_L and V_H domains. An α-carbon trace is shown with side chains of residues that differ between the murine and humanized versions. The six CDRs are labeled, and the side chains of V_L R53 and V_H K73 are represented by space-filling balls.

of chimeric BsF(ab')$_2$ to enhance the cytotoxic activity of CTL with each of the BsF(ab')$_2$ variant molecules. The addition of 10 ng/ml of chimeric BsF(ab')$_2$, BsF(ab')$_2$ v1, or v3 caused a three- to fourfold enhancement of the cytotoxicity of CTLs against SK-BR-3 breast tumor cells (Fig. 5 *A*) whereas the presence of BsF(ab')$_2$ v2 or v4 resulted in no enhancement above control values. Enhancement caused by BsF(ab')$_2$ v1 was reversed by the addition of p185^{HER2} ECD to the assay mixture (Fig. 5 *A*) demonstrating the specificity of antibody action. The results from an independent experiment (Fig. 5 *B*) demonstrate that 10 ng/ml of BsF(ab')$_2$ v1 consistently enhanced the function of CTLs against SK-BR-3 targets known to overexpress p185^{HER2} (64 pg/μg cell protein; 3, 12) but had no effect on the cytotoxicity of CTLs against MDA-MB-175 targets, which express low to moderate levels of p185^{HER2} (7.7 pg/μg cell protein; 3, 12). These results demonstrate the efficacy of chimeric BsF(ab')$_2$ v1, and v3 in directing cytotoxic lymphocytes to kill breast tumor targets overexpressing p185^{HER2} but not targets with low p185^{HER2} expression. The cytotoxicity data (Fig. 5 *A*) correlate well with FACS® binding data (Fig. 4) in that BsF(ab')$_2$ v2 and v4, which were inefficient in binding to cytotoxic lymphocytes, failed to direct tumor cell killing in the cytotoxicity assay. None of these BsF(ab')$_2$ molecules affected the cytotoxic activity of human CTL when tested against PHA-induced blastogenic targets derived from the same donor, demonstrating that these BsF(ab')$_2$ do not mediate the lysis of normal autologous lymphoid cells.

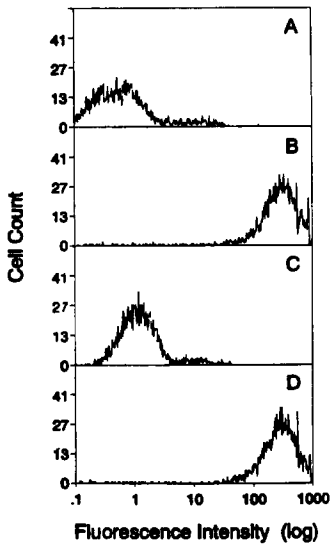

Figure 3. Flow cytometric analysis of NR6/10 cells bound to chimeric BsF(ab')$_2$. Cells were incubated with PBS (*A*), chimeric BsF(ab')$_2$ (*B*), chimeric BsF(ab')$_2$ mixed with p185^{HER2} ECD (*C*), or chimeric BsF(ab')$_2$ mixed with recombinant CD4 (*D*) before staining with goat anti-human F(ab')$_2$-FITC (conjugated Ab).

Discussion

Considerable progress has been made toward the development of BsmAbs as therapeutic agents for human cancer (reviewed in reference 9). Human CTLs directed with BsmAb

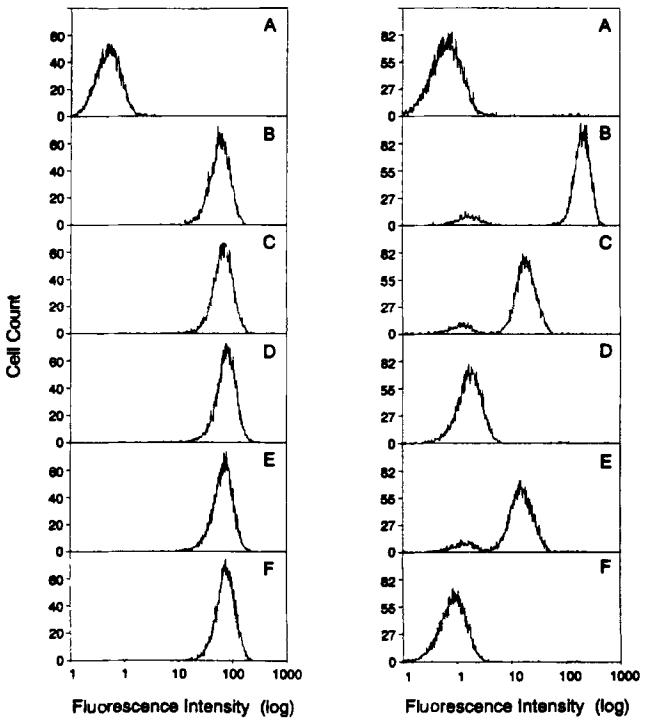

Figure 4. Flow cytometric analysis of the binding of $BsF(ab')_2$ antibodies to human CTLs and human breast tumor SK-BR-3 cells. Histograms on the *left* illustrate SK-BR-3 cells incubated with PBS (*A*), chimeric $BsF(ab')_2$ (*B*), $BsF(ab')_2$ v1 (*C*), v2 (*D*), v3 (*E*), or v4 (*F*) before staining with the FITC-conjugated Ab. Histograms on the *right* illustrate human CTLs incubated with PBS (*A*), chimeric $BsF(ab')_2$ (*B*), $BsF(ab')_2$ v1 (*C*), v2 (*D*), v3 (*E*), or v4 (*F*) before staining with FITC conjugated Ab.

have been shown to block the growth of human tumor xenographs in nude mice (31, 32). In other studies involving carcinoma patients, local lysis of tumor cells was observed after infusion of T cells activated with BsmAb (8). In addition, the efficacy of antitumor associated antigen × anti-CD3-bispecific antibody in the management of malignant glioma has been reported (33). A major drawback for the application of murine mAbs has been the elicitation of an immune response after repeated administration into humans. The humanization of BsmAb may reduce the immunogenicity of these reagents, thus avoiding possible untoward effects in human subjects (10, 11, 34).

The development of biologically active fully humanized $BsF(ab')_2$ fragments as demonstrated in this study has not been reported previously. We have used an efficient *E. coli* expression system (19) for the production of humanized Fab' molecules with anti-$p185^{HER2}$ and anti-CD3 specificities. The Fab' molecules were recovered with the unpaired hinge cysteine present as the free thiol and used to form the $BsF(ab')_2$ by directed chemical coupling in vitro (29). The approaches used in this study obviate the inherent problems in generating Fab'-SH from intact antibodies: differences in susceptibility to proteolysis and nonspecific cleavage resulting in heterogeneity, low yield, as well as partial reduction that is not completely selective for the hinge disulfide bonds (29). Furthermore, by engineering the hinge region to leave a single cysteine residue, we prevent intrahinge disulfide bonding without resorting to the use of highly toxic arsenite to chelate vicinal thiols (29). The purified $BsF(ab')_2$ antibody fragments are reactive with human T cells and cells overexpressing $p185^{HER2}$. The ability of these $BsF(ab')_2$ to mediate targeted killing of tumor cells correlated well with their efficiency of binding to CTLs as revealed by FACS® analysis.

The humanization of the anti-CD3 arm (as in v1) resulted in a decrease in the binding efficiency to CTL but did not alter the ability of the molecule to enhance CTL cytotoxicity against tumor targets at the lowest $BsF(ab')_2$ concentration studied (10 ng/ml). The observation that <2% occupancy by antibody is sufficient to trigger T cell activation (35) together with the high degree of purity of the $BsF(ab')_2$ used may explain the observed potency of the humanized version in mediating tumor cell killing at pharmacological concentrations (10 ng/ml).

Replacement of the murine residue V_L R53 with serine

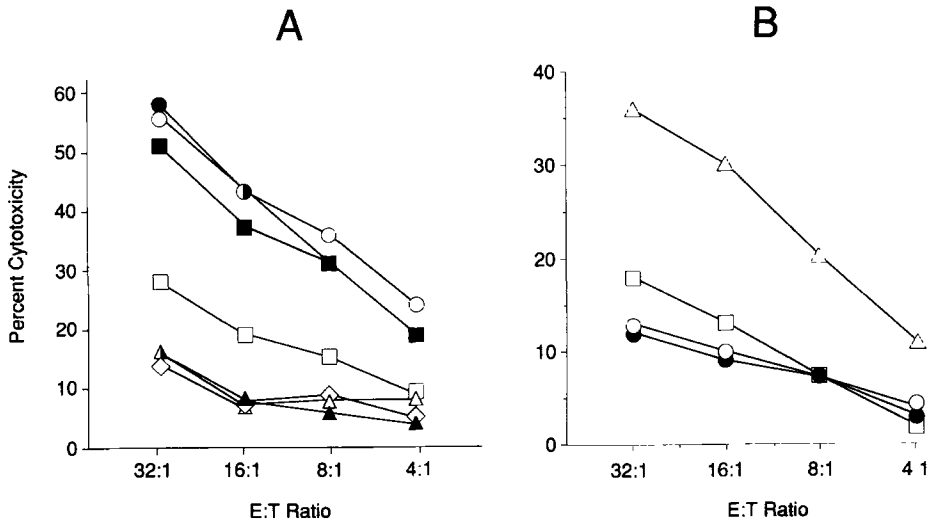

Figure 5. Targeting of breast tumor cell killing by $BsF(ab')_2$. ^{51}Cr-labeled SK-BR-3 targets (*T*) were co-incubated with effector (*E*) CTLs at different E/T ratios for 4 h. In *A*, percent cytotoxicity was calculated based on ^{51}Cr release in cultures with no antibody added (▲), in the presence of 10 ng/ml of chimeric $BsF(ab')_2$ (●), $BsF(ab')_2$ v1 (○), v2 (◇), v3 (■), v4 (△), or v1 + $p185^{HER2}ECD$ (□). In *B*, two different ^{51}Cr-labeled target cells were used. MDA-MB-175 targets tested in the absence (○) or presence (●) of 10 ng/ml of $BsF(ab')_2$ v1; and SK-BR-3 targets tested in the absence (□) or presence (△) of 10 ng/ml of $BsF(ab')_2$ v1. The MDA-MB-175 cells express low to moderate level of $p185^{HER2}$ as quantitatively stated in Results.

(human residue) in humanized anti-CD3 v1 to create v3 resulted in little or no change in the binding efficiency to CTL, suggesting that V_L R53 is probably not an essential antigen-binding determinant. The binding efficiency of v1, however, was severely reduced upon the replacement of V_H K73 with the human counterpart, Asp, in FR3 to make v2. Further reduction of the binding to CTLs was observed when V_L R53 in v2 was replaced by Ser to create v4, whose binding capacity to CTLs was almost completely abolished. Given the fact that V_H K73 is outside of the CDRs, these results imply that contact between selected amino acid residues in FRs with other residues in adjacent CDRs or direct interaction with antigen can influence the antigen-binding efficiency and specificity of the hypervariable loops (11, 36). However, additional amino acid replacements are required to determine whether murine residue V_H K73 is an important binding determinant or whether the human residue D73 compromises binding. Nevertheless, these data demonstrate that amino acid residues outside CDRs should be considered in mAb humanization. Additional amino acid replacements are currently being installed in the humanized anti-CD3 Fab' in an effort to improve its binding efficiency. A three-dimensional molecular model of the humanized (v1) anti-CD3 arm V_L and V_H domains is presented (Fig. 2), illustrating the side chains of residues that differ between murine and humanized versions of anti-CD3 arm. This figure shows that V_H K73 in FR3 is located in a loop proximal to CDRs H1 and H2.

The fact that a number of adenocarcinomas are characterized by an overexpression of $p185^{HER2}$ presents a unique opportunity for testing the feasibility as well as the efficacy of targeted tumor immunotherapy whereby patients CTLs can be redirected with BsmAb for tumor killing. Fully humanized $BsF(ab')_2$ fragments are shown here to be effective mediators of human breast tumor target lysis in vitro at pharmacologically relevant concentrations. We are currently investigating whether these molecules may be capable of targeting breast tumor cells in vivo for destruction by CTLs. The fact that these molecules are fully humanized and, therefore, less likely to elicit an immune response in cancer patients further advances their potential use in targeted immunotherapy. Importantly, the $BsF(ab')_2$ molecules failed to mediate the killing of normal lymphoid cells (PHA-blasts) or even tumor cells expressing only low to moderate levels of $p185^{HER2}$. The mechanism(s) by which CTLs cause the lysis of tumor targets are not known. However, it has been reported that the cytolytic activity is induced by the binding of antibody-target conjugates to specific receptors on the effector cell surface (6). One view proposes that the crosslinking by BsmAbs between clusters of receptors on tumor targets and triggering structures on immune effector cells can induce the release of effector cytolytic substances including cytolysin which may contribute to target killing (8). Crosslinking may also activate T cells for production of cytokines, e.g., TNF-α and IFN-γ, both of which can exert cytotoxic effects on tumor cells in vitro. The data in this report are consistent with the crucial requirement for crosslinking in order to trigger effector killing of tumor target. Thus two of the $BsF(ab')_2$ variants, v2 and v4, shown to be perfectly capable of binding to target cells but not to effector CTLs, failed to direct tumor killing in the cytotoxicity assay. Further, the presence of $p185^{HER2}$ ECD, which is shown to block $BsF(ab')_2$ binding to the target, caused a marked inhibition of directed CTL killing of tumor targets.

Our *E. coli* Fab' expression system in combination with directed chemical coupling as described here has proven effective in the production of clinically relevant quantities of functional humanized antibody fragments. The availability of purified material should facilitate the initiation of clinical studies to evaluate the efficacy of $BsF(ab')_2$ in redirecting CTL killing of tumor cells. It should be noted that the use of $F(ab')_2$ fragments should permit for a more efficient tissue penetration in vivo (37). The systems used here also allow for replacements of amino acid residues in CDRs and FRs making it possible to study structure-function relationships among the different variant Ab fragments.

Collectively, the data presented here demonstrate the feasibility of producing genetically engineered fully humanized $BsF(ab')_2$ shown to be biologically active in two different in vitro assays. The expression systems described can be applied efficiently for the production of Fab' molecules with selected specificities, and offer an opportunity for understanding the structure-function relationship among the produced Ab fragments. These and similar studies will advance the potential use of BsmAbs in targeted immunotherapy of cancer in humans.

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