**In Vitro Cytotoxic Targeting by Human Mononuclear Cells and Bispecific Antibody 2B1, Recognizing c-erbB-2 Protooncogene Product and Fcγ Receptor III**

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**ABSTRACT**

Bispecific murine monoclonal antibody 2B1, possessing dual specificity for the human c-erbB-2 protooncogene product and human Fcγ receptor III (CD16) was evaluated for the ability to promote specific lysis of c-erbB-2-positive tumor cells *in vitro*. In short-term ³⁵⁰Cr release assays with human mononuclear cells as effectors and SK-Br-3 human breast cancer cells as targets, neither parental antibody of 2B1 mediated significant specific lysis, but bispecific antibody was as active as a chemical heteroconjugate, with 5 ng/ml of 2B1 causing half-maximal lysis at an effector/target ratio of 20:1 and 2 ng/ml 2B1 causing half-maximal lysis at an E/T ratio of 40:1. The cytotoxic targeting activity of 2B1 F(ab′)₂ fragment was the same as that of whole bispecific antibody, and the activity of whole 2B1 was not reduced when assays were performed in 100% autologous human serum, indicating that 2B1 binds effector cells through the CD16-binding site derived from parental antibody 3G8 rather than through its Fc portion. Variable inhibition of 2B1 bispecific monoclonal antibody was also able to mediate targeted cytolyis using whole human blood as a source of effector cells or using effector or target cells derived from ovarian cancer patients.

**INTRODUCTION**

Targeted cytolysis of tumor or virus-infected cells occurs when effector cells are directed by antibodies with simultaneous specificities for both targets and effectors. Agents that have been successfully used to target cytotoxicity include chemically linked AHChs,² as well as BsMAbs produced by hybrid hybridomas (1). The latter have the advantage of being smaller in size; therefore, trafficking to tumor sites may be easier and clearance by the reticuloendothelial system may be slower (2). BsMAbs are easier to manufacture once stable clones of hybrid hybridomas are established, and any possibility of instability in the chemical linkage between the two antibody molecules is also avoided. (3, 4).

Effector cells that have been used in targeted cytotoxicity using BsMAbs include cytotoxic T-lymphocytes, monocytes, macrophages, and LGLs or natural killer cells. Antibodies against CD3 (5–7) as well as T-cell receptor γδ (8) have been used as the effector arm of BsMAbs targeting T-lymphocytes against ovarian (9–11) as well as renal carcinomas (6). Natural killer cells or LGLs have been targeted to lyse colorectal (12) or ovarian (13) tumor cells *in vitro* with a BsMAb having an anti-FcγR III antibody as the effector arm, and others have reported successful targeting of monocytes or macrophages with anti-FcγR I AHCs (14).

In our laboratory, we have made a panel of monoclonal antibodies that react with the c-erbB-2 protooncogene product p185 (15, 16). Monoclonal antibody 520C9 was ineffective in classical ADCC lysis with human peripheral blood lymphocytes, probably because of its IgG1 isotype. However, when 520C9 was chemically conjugated to anti-FcγR III antibody 3G8, strong targeted cytolysis of cell lines overexpressing p185 was observed. Based on these encouraging results, we proceeded to fuse the hybridoma pair 520C9 and 3G8 and obtained a stable hybrid hybridoma clone named 2B1 (17). In this paper, we examine the ability of 2B1 BsMAb to mediate targeted cytotoxicity with human effector cells from both healthy donors and ovarian cancer patients.

**MATERIALS AND METHODS**

Antibodies. Murine IgG1x monoclonal antibody 520C9 was raised against membranes from a human breast carcinoma (18) and recognizes the extracellular domain of the human c-erbB-2 protooncogene product p185 (15). Murine IgG1x monoclonal antibody 3G8 was obtained from Dr. Jay Unkeless (Mt. Sinai School of Medicine, New York) and recognizes human FcγR III or CD16 (19, 20). Monoclonal antibodies were purified from BALB/c ascites by size exclusion and ion exchange chromatography.

Hybrid hybridoma 2B1 was produced by fusing hybridomas 520C9 and 3G8, as previously described (17). Hybrid hybridoma 1A7 was similarly produced by fusing hybridomas 15D3 (anti-human P-glycoprotein) and 3G8 (21). BsMAs produced by these hybrid hybridomas were purified from serum-free culture medium by anion and cation exchange chromatography. The 520C9–3G8 heteroconjugate was cross-linked with N-succinimidyl 3-(2-pyridyldithio)propionate (22). 2B1 F(ab′)₂ fragment was produced by papain digestion and purified by size exclusion and protein A affinity chromatography (21).

Target Cells. Human cancer cell lines BT-20, BT-474, MCF-7, MDA-MB-175 VII, and MDA-MB-361 were obtained from the Mason Research Institute (collection now transferred to American Type Culture Collection). SK-Br-3 and CaMa-1 were gifts from Dr. J. Fogh (Sloan Kettering Memorial Institute). CaLu3, K562, and SW948 were obtained from the American Type Culture Collection (Rockville, MD). OVCAR3 was obtained from Dr. Thomas C. Hamilton (National Cancer Institute), and normal human foreskin fibroblast line CC95 was a gift from David Buck (Becton Dickinson, Mt. View, CA). All cell lines were cultured in Iscove’s modified Dulbecco’s medium plus 2 mm glutamine and 10–15% heat-inactivated fetal bovine serum, and all tested negative for *Mycoplasma* contamination.

For chromium release assays, target cells were dislodged from the flask with 1:5000 versene, washed, and labeled with 122 μCi of ⁵¹Cr (300–500 μCi/mg; NEN, Boston, MA) in 1 ml Hanks’ balanced salt solution for 60 min at 37°C, washed extensively, and plated at 20,000 cells/well in V-bottom 96-well microtiter plates (Costar, Cambridge, MA). For tritium release assays, a subconfluent flask of target cells was labeled with 62.5 μCi of [³H]thymidine (6.7 Ci/mmol; NEN) and cultured for 1–7 days, depending on the growth rate of the cell line.
Preparation of Effector Cells. Heparinized human blood was obtained from healthy donors, and TMC were separated by differential centrifugation in Ficoll-Hypaque (Histopaque 1077; Sigma). To isolate nonadherent mononuclear cells, TMC were allowed to adhere to culture flasks during a 30-min incubation at 37°C, and nonadherent cells were then collected. To isolate PMNs, the pelleted PMNs and RBCs obtained from Ficoll-Hypaque centrifugation were mixed with 1.5% dextran (M, 229,000; Sigma) and allowed to settle for 30–60 min at 37°C. The supernatant containing the PMNs was then collected. Effector cells used in all experiments showed viability of >95% by trypan blue dye exclusion.

Cells from Ovarian Cancer Patients. Fresh human ovarian ascites was obtained with human subjects committee approval and provided by Dr. John Donohue (Mayo Clinic, Rochester, MN). RBCs and dead cells were removed by Ficoll-Hypaque differential centrifugation, and viable cells were resuspended in a medium containing 62.5 μCi (3H)-thyminidine and cultured for 3–4 days. Heparinized blood or total mononuclear cells from ovarian cancer patients were also obtained from Dr. John Donohue and processed using the same procedures as for normal donor blood.

Chromium Release Assay. The medium used in the assay was Isco’s with 2 mm glutamine, 50 μg/ml gentamicin, and 10% heat-inactivated human serum from the same donor as the effector cells (autologous serum). Chromium-labeled target cells were added to antibody dilutions in microtiter wells and incubated 60 min at 37°C. The plates were then spun 15 min at 200 × g, and the antibody dilutions removed before effector cells were added at desired E/T ratios. After 3 h incubation at 37°C, the plates were spun again, half the content of the wells (100 μl) was collected, and chromium release was determined in a gamma counter. Each sample point was done in triplicate, and results are shown as mean ± SD. The percentage of specific lysis was calculated as

\[
\text{Percentage of specific lysis} = \left( \frac{\text{Mean sample release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \right) \times 100
\]

Spontaneous release was measured from target cells in assay medium alone, and maximum release was measured after lysis in 1% Nonidet P-40.

Tritium Release Assay. Effector cells were dispensed into 96-well round-bottom tissue culture plates in AIM V medium (Gibco) with 8 mm glutamine and 5 units/ml IL2 (final concentration) and with or without 10% heat-inactivated autologous serum. After 3 days of preincubation, dilutions of antibodies and tritium-labeled target cells were added. Tritium released in the supernatant was measured after 3 more days of incubation. Each sample was tested in triplicate in each experiment. Percentage of specific lysis was calculated as in the chromium release assay protocol. Maximum release was measured after lysis in 1% sodium dodecyl sulfate.

Whole Blood Cytolytic Assay. Heparinized whole blood was dispensed into 48-well tissue culture plates at 0.5 ml/well, followed by 0.25 ml/well antibody (final concentration, 1 ng/ml) and 0.25 ml/well tritium-labeled target cells. Tritium release was measured after 3 days of incubation as in the standard tritium release assay protocol.

Bridge Assay. Total mononuclear cells were stained with rhodamine-123 (final concentration, 4 μg/ml; Eastman Kodak, Rochester, NY) for 10 min at 37°C. SK-Br-3 cells were labeled with hydroethidine (10 μg/ml; Polysciences, Warrington, PA) for 20 min at room temperature. Both cell types were washed extensively in Hanks’ balanced salt solution before being resuspended together in antibodies diluted in various concentrations of autologous serum. The cells were then centrifuged at 50 × g for 2 min, followed by incubation at 4°C for at least 30 min. Doubly fluorescent complexes were analyzed on an Epics V cell sorter. The bridging effect was calculated as the percentage of tumor cells found in doubly fluorescent complexes.

Cell Surface Antibody Staining. One million target cells were first incubated with 5 μg/ml 2B1 or 520C9 for 30 min at 4°C, washed, and then stained with 10 μg/ml fluorescein isothiocyanate-conjugated goat F(ab')2 anti-mouse IgG (Fc specific; Jackson ImmunoResearch, West Grove, PA) for another 30 min at 4°C. Live cells (20,000) were gated by forward angle light scatter and propidium iodide and scored using a 525-nm bandpass filter on a Coulter EPICS 5 cell sorter.

RESULTS

Correlation of 2B1-targeted Cytolysis with c-erbB-2 Expression by Target Cells. 2B1 BsMab was generated after exploratory experiments indicated that chemically linked 520C9–3G8 heteroconjugates were able to promote lysis of c-erbB-2-expressing cell lines. The 2B1 hybrid hybridoma produced parental antibody 520C9, bispecific antibody, and parental antibody 3G8, which eluted sequentially on anion exchange chromatography. An inactive form of bispecific antibody (differing in glycosylation) was removed by cation exchange chromatography, and the remaining active 2B1 BsMab was shown to bind c-erbB-2-positive SK-Br-3 cells and CD16-positive human PMNs (21). No evidence was seen that the 2B1 hybrid hybridoma produced any inactive immunoglobulin species resulting from light/heavy chain mispairing (21).

Purified active 2B1 BsMab was tested on a panel of c-erbB-2-positive or -negative cell lines to compare the level of targeted cytolysis with the presence or absence of target antigen expression (Table 1). Results from 2B1-mediated cytolysis using human TMC at a 20:1 E/T ratio showed a general correlation with immunofluorescent cell-binding data. In the presence of 1 μg/ml 2B1, c-erbB-2-positive tumor targets, including breast, colorectal, and ovarian cancer cell lines, showed 18–44% 2B1-dependent lysis in a 3-h 51Cr release assay above the level observed in the absence of antibody, while negative or weakly staining cell lines showed 4–10% lysis. The results in Table 1 were confirmed in various experiments using c-erbB-2-positive or -negative cell lines with either human TMC or cultured human monocytes as effectors (data not shown). Consistent with its high expression of c-erbB-2 (23, 24), the SK-Br-3 cell line was particularly susceptible to 2B1-targeted lysis and was used for most of the experiments described below.

Targeted Cytolysis by 2B1-bispecific Antibody versus Parental Antibodies and Other Controls. Fig. 1 shows dose-response curves comparing lysis mediated by 2B1 BsMab or chemically linked 520C9–3G8. Both the BsMab and the heteroconjugate

<table>
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<th>Target Cell</th>
<th>c-erbB-2 expression</th>
<th>% lysis, medium only</th>
<th>% lysis, 1μg/ml 2B1</th>
<th>% 2B1-mediated cytosis</th>
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<tr>
<td>Breast cancer</td>
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<td></td>
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<tr>
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<td>CC95</td>
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Table 1: Correlation of target cell c-erbB-2 expression and 2B1-mediated cytosis

Binding of 2B1 or of parental antibody 520C9 was analyzed by immunofluorescent flow cytometry according to the protocol described in "Materials and Methods" or by indirect immunofluorescent staining of live cells on slides as previously described (18). Staining by either method was scored positive (+), weak, or negative (−). Targeted cytolyis was evaluated using 1 μg/ml 2B1 BsMab with total human mononuclear cells and chromium-labeled target cells at an E/T ratio of 20:1. Percentage of 2B1-mediated lysis was calculated as (% specific lysis with 2Bl) − (% specific lysis without antibody).

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mediated 50–60% maximal lysis of the target breast cancer cell line SK-Br-3; BsMAb caused half-maximal lysis at about the same concentration as heteroconjugate, a result confirmed in eight other experiments not shown. The two parental antibodies of 2B1, anti-c-erbB-2 antibody 520C9 and anti-human FcγR III antibody 3G8, did not mediate specific lysis alone or when mixed together (10 experiments). The negative control BsMAb IA7, recognizing human P-glycoprotein and human FcγR III, also did not mediate lysis of SK-Br-3 (22 experiments), although it does lyse multiple drug-resistant cell lines (data not shown).

The median concentration of 2B1 that caused half-maximal lysis of 51Cr-SK-Br-3 in 18 experiments using E/T ratios of 15:1 to 25:1 was 5.4 ng/ml; in another 14 experiments using an E/T ratio of 40:1, the median concentration causing half-maximal lysis was 2.1 ng/ml. Fig. 2 shows a representative assay performed at a 20:1 E/T ratio. All of these experiments were done in 5–10% heat-inactivated autologous human serum, using SK-Br-3 cells as targets and TMC as effectors. Fig. 2 also shows that the 2B1 F(ab')2 fragment mediated SK-Br-3 lysis as effectively as whole 2B1, despite the removal of the Fc portion of the bispecific antibody molecule; similar results were observed in 10 experiments using effector cells from 5 different donors.

Role of Adherent Cells in Cytolysis. To investigate the possible contribution of monocytes to 2B1-mediated cytolysis, in some assays monocytes were removed from TMC by adherence to plastic tissue culture flasks. Nonadherent mononuclear cells were able to target cytolysis of SK-Br-3 cells as well as TMC at E/T ratios as low as 5:1 (Fig. 3). Comparable activity of total versus nonadherent mononuclear cells was observed in six experiments using effector cells from five donors. Since CD16-positive NK/LGL cells constitute only about 15% of peripheral blood mononuclear cells (19), the relevant E/T ratio in these experiments was approximately 15% of the nominal E/T ratio.

Inhibition of 2B1-mediated Cytolysis in the Presence of PMNs. Because 3G8 also binds PMNs strongly (25) and since PMNs are abundant in peripheral blood, we examined 2B1-targeted cytolysis using TMC from nine donors in the presence or absence of a 2:1 ratio of autologous PMNs. Since the observed effects varied both qualitatively and quantitatively from donor to donor. Fig. 4 shows the results of three representative experiments. When effector cells from donor 269 were used, the addition of PMNs resulted in a substantial reduction in target cell lysis, which was only partly overcome at the highest 2B1 concentration tested (10 μg/ml). For donor 140, PMNs caused a 10–35% reduction in cytolysis at 2B1 concentrations ranging from 1 ng/ml to 10 μg/ml. On the other hand, for donor 230, the inhibitory effect of PMNs was completely overcome at 1 μg/ml 2B1; this last experiment used a slightly higher E/T ratio (50:1 versus 40:1) and a longer lysis period (overnight instead of 3 h).

Cytolytic Targeting by 2B1 in the Presence of Serum Immunoglobulin. The effects of human serum on 2B1-targeted cytolysis were studied in two ways: first, by observing the ability of 2B1 to complex TMC with SK-Br-3 cells in various concentrations of autologous serum (Fig. 5a) and, second, by performing targeted cytolysis in 100% autologous serum instead of 10% autologous serum in standard assay medium (Fig. 5b). Fig. 5b is representative of four cytotoxicity experiments performed with cells from different donors. Results from both types of experiment showed that 100% autologous serum had no noticeable blocking effect on either 2B1-mediated cell complexing or cytolysis. Similarly, 100% autologous serum did not reduce binding of 2B1 BsMAb to human PMNs or LGLs (data not shown).

To further study whether 2B1 could mediate lysis in the presence of all blood components found in vivo, whole blood
Monoclonal antibody 520C9 was originally selected on the basis of moderately broad binding to a variety of carcinoma types and very restricted binding to normal human tissues (16). We later came to suspect that the approximately Mr 200,000 glycoprotein recognized by 520C9 was the c-erbB-2 oncogene product p185. This identity was confirmed by showing that 520C9 recognized a truncated recombinant form of p185 (15).

**Expression of c-erbB-2 in Human Tumors.** Expression of protooncogene c-erbB-2 in fresh human tumors and tumor cell lines has been studied extensively (23, 24, 26–29). Thirty to 40% of human breast, ovarian, and colon tumors have been found to express the c-erbB-2 product, p185 (28, 30). Correlations between amplification of the c-erbB-2 gene and/or overexpression of p185 in breast and ovarian tumors and poor prognosis for the patients have been investigated by various groups (31–35). Other human tumors and cell lines that have been associated with overexpression of the c-erbB-2 gene or p185 include neuroblastoma (36), lung cancer (26, 37), thyroid cancer (38), pancreatic cancer (39), and cancers of the digestive tract (39, 40).

**Effects of Antibody Binding to p185.** Localization to or growth inhibition of p185-positive tumors with monoclonal antibody 520C9 was achieved in various studies. A significant finding was the ability of the antibody to mediate killing of p185-positive cells in several tumor cell lines and primary tumors. The ability of antibody to mediate lysis of p185-positive cells in the absence of complement was confirmed in a number of studies. The mechanism of lysis was not studied, but it is possible that the antibody-directed cells were killed by antibody-dependent cellular cytotoxicity (ADCC) or by a direct effect on the tumor cells. The results of these studies suggest that antibody to p185 may be a useful therapeutic agent for the treatment of cancers expressing this oncogene.
Antibodies has recently been reported (41-47). Anti-p185 MAbS have been shown to inhibit growth of c-erbB-2-positive tumor cells directly, and also by complement-mediated lysis, by classical ADCC or by rendering tumor cells more susceptible to cytokine mediators such as TNFα or to the alkylating agent cis-diaminedichloroplatinum (41, 42, 44, 46, 48). In this paper, we have shown that a number of cancer cell lines were immuno-reactive with our 2B1 BsMAb and/or its parental antibody 520C9 and were lysed by 2B1 in targeted cytolyis assays. These same cell lines have been found by other groups to be c-erbB-2 positive with elevation of protein and mRNA levels as well as gene amplification (23, 24, 47).

**Bispecific Antibody Activity in Vitro.** Several factors are likely to influence the efficiency of BsMAb-targeted cytotoxicity targeted in vitro. Among these are (a) the density and uniformity of target antigen expression on target cells and triggering receptor expression on effector cells, (b) the susceptibility of a particular target cell to lysis by a given effector cell type, (c) the kinetics of lysis by a particular effector cell type in relation to the length of the assay protocol used, and (d) the E/T cell ratio.

Density of antigen and triggering receptor expression is likely to affect the concentration of BsMAb required for half-maximal cytolytic activity. This concentration is presumably related to the binding constants for the two antibody specificities but in a complicated fashion, since the cell complexing that leads to lysis is a highly multivalent event. The concentration of 2B1 required for half-maximal lysis of SK-Br-3 target cells at E/T ratios of 20:1 to 40:1 was 2–5 ng/ml or 13–33 pM, far below the $K_d$ of 35 nM previously reported for bivalent 520C9 (16). The difference, again, is likely to be explained by multivalent interactions between the complexed cells, which would be expected to drastically reduce the dissociation rate for bound BsMAb.

Uniformity of target antigen expression and target cell susceptibility to lysis are likely to have significant effects on the maximal lysis observed, as is the relationship between assay length and lysis kinetics. Some target cells may escape lysis because they fall below a critical level of target antigen density and others because they are innately more resistant to lysis (e.g., by expressing lower levels of a cell adhesion ligand that synergizes in triggering a cytolytic event). Most of the experiments reported in this paper were 3- to 4-h $^{3}H$ release assays. Such short assays may not allow time to observe lysis of all target cells, and in fact, we uniformly observed higher maximal lysis using 2B1 in 3-day $^{3}H$ release assays.

Higher E/T cell ratios are likely to produce higher levels of maximal lysis and may also reduce the concentration of BsMAb required to achieve a given level of lysis, possibly by increasing the number of available effector cells with higher levels of triggering receptor. Consistent with this expectation, we observed half-maximal lysis at 2 ng/ml 2B1 in experiments done at a 40:1 E/T ratio versus 5 ng/ml 2B1 in experiments done at an approximately 20:1 E/T ratio.

Only a limited number of studies have been carried out with CD16-directed BsMAbs. Garcia de Palazzo et al. (12) reported a BsMAb derived from 3G8 and antitumor monoclonal antibody CA19-9, while Ferrini et al. (13) reported a BsMAb directed to CD16 and the MOV19 ovarian cancer surface antigen. It is difficult to make close comparisons of these studies with our own work because of variations in effector cell type, target cell/antigen type, and assay conditions. Comparing 3- to 4-h chromium release assays performed by each group, Garcia de Palazzo et al. observed half-maximal lysis of SW948 colorectal cancer cells at approximately 0.75 ng/ml BsMAb using IL-2-stimulated human PBLs at a 5:1 E/T ratio; Ferrini et al. observed half-maximal lysis of IGROV-1 ovarian cancer cells at about 22 ng/ml BsMAb using the CK8 NK cell line at a 2:1 E/T ratio; as mentioned above, we observed half-maximal lysis of...
SK-Br-3 breast cancer cells at 2–5 ng/ml 2B1 BsMAb using unactivated human TMC at a 40:1 or 20:1 E/T ratio. Given the disparities in the assays, these results are in reasonable agreement. Both of the other groups observed somewhat higher lysis at low E/T ratios (<5:1) than we did with 2B1 BsMAb; the difference may be related to issues of BsMAb affinity and target antigen copy number or to the fact that Garcia de Palazzo et al. used IL2-treated PBLs and Ferrini et al. used an NK cell clone rather than unactivated TMC.

Circumstances Likely to Affect Bispecific Antibody Activity in Vivo. An additional set of factors may influence cytotoxic targeting by BsMabs in vivo. Among these are (a) the ability of BsMAb to promote lysis in the presence of normal serum immunoglobulin; (b) the number, competence, and activation state of available effector cells, particularly in patients who may be immunocompromised by other forms of therapy; (c) the biodistribution of BsMAb to tumor cells, to normal cells bearing the same epitope, to effector cells, to other normal cells bearing Fc receptors, and to routes of clearance; (d) the trafficking of effector cells once decorated with BsMAb; and (e) the development of host antibody response against recognizably foreign BsMabs.

The use of monoclonal antibodies that mediate classical Fc-dependent ADCC in treating patients may be hampered by the high concentration of IgG in human serum, which can compete with the MAB for binding to Fc receptors on the effector cells. This is most likely for ADCC involving the high-affinity Fcγ receptor I, which can be tightly blocked by monomeric immunoglobulin. Using bifunctional antibodies that have one arm directed against an Fc receptor is expected to circumvent any such problem. The effector arm of our 2B1 BsMAb is 3G8, which recognizes low-affinity Fcγ receptor III. Although 3G8 is reported to react with an epitope of CD16 on or close to the ligand (Fc)-binding site (25), serum immunoglobulin did not interfere with the binding of 2B1 to the effector cells we used. When we carried out targeted cytotoxicity assays with 2B1 in 100% human serum or in whole blood, we observed undiminished specific lysis, while classical ADCC mediated by the IgG3 monoclonal antibody 113F1 (which could be expected to bind to FcγR I) was blocked by only 10% human serum (data not shown). Furthermore, the F(ab′)2 fragment of 2B1 mediated targeted lysis as well as whole 2B1, clearly demonstrating that the Fc portion of 2B1 need not bind to effector cell receptors.

Since the effector cell binding arm of 2B1 BsMAb recognizes CD16, we expect to recruit a variety of effector cells including NK cells or LGLs, PMNs, macrophages, and certain monocytes, as well as a subset of γ/δ T-cells (13, 49). Natural killer cells or LGLs have been shown to be good effectors in targeted cytotoxicity by other groups using AHCs as well as BsMabs (12, 13, 50, 51). Using NK cells as effectors in heteroconjugate-mediated cytolyis, Qian et al. (52) found that direct killing could be observed in short 3- to 4-hour 51Cr release assays, while cytostasis mediated by cytokines could only be evaluated when longer assay protocols were used. Trinchieri et al. (49) reported that NK cells under proper tumor cell stimulation released cytokine mediators such as TNFa to inhibit tumor growth. C-erbB-2-positive cell lines such as the SK-Br-3 line used in our experiments are known to be resistant to TNFa (53), but their resistance could be overcome by adding anti-c-erbB-2 antibody (48). In our experiments, 2B1 may be involved both in bringing effectors and target cells together for direct killing and also in rendering target cells more susceptible to cytokine-mediated cytosis or cytostasis.

In addition to NK cells and LGLs, the other CD16-positive effector cells in our total mononuclear cell preparation were monocytes. Monocytes constitute about 10% of the mononuclear cells, but only 20% or less of fresh monocytes are CD16 positive (54). In our usual protocol using a TMC to target cell ratio of 20:1, the actual monocyte to target ratio would have been only 0.4:1, which was below the level at which we can observe 2B1-mediated targeted cytotoxicity by purified adherent monocytes (data not shown). Very similar levels of 2B1-mediated lysis were observed whether or not monocytes were removed by adherence, indicating a limited role for monocytes in the results shown in this paper. When purified fresh or cultured monocytes were used as effector cells in 2B1-mediated cytosis, measured by tritium release assays, specific lysis was seen.

PMNs are the third and largest group of CD16-positive effector cells in peripheral blood. Recently, PMNs were reported to lyse a neuroblastoma cell line and neuroectodermal tumor cells using mouse anti-GD2 monoclonal antibodies in ADCC assays (55, 56). When we used dextran-purified PMNs in our targeted cytotoxicity assay, we failed to observe any 2B1-mediated cytotoxicity. However, when we used hypotonic shock or a modified Ficoll-Hyapaque method (Histopaque 1119, Sigma) to isolate the neutrophils, we observed a small amount of specific lysis at 1–10 µg/ml 2B1 with PMNs from some donors (data not shown). Other investigators have also reported targeted tumor lysis by PMNs with a 3G8 heteroconjugate but only at a high AHC concentration of 1 mg/ml (22). PMNs are known to shed FcγR III when activated (57–59), and it is possible that purification processes such as dextran sulfate precipitation or Ficoll-Hyapaque differential centrifugation may activate PMNs enough to cause loss of FcγR III. Accordingly, tumor cell lysis by purified PMNs may be difficult to observe in vitro.

Since PMNs can bind 2B1 very well, but do not contribute significantly to tumor lysis, they could not only compete with NK cells/LGLs for 2B1 binding in vivo but also could occlude the surface of 2B1-decorated targets and prevent NK cells/LGLs from complexing with tumor cells. To evaluate this possibility, we added PMNs to total mononuclear effector cells in our assay and found only partial blocking of targeted lysis. The amount of blocking varied from donor to donor and also from experiment to experiment, presumably depending on the number as well as the activation states of the PMNs and LGLs. The ratio of PMNs to TMC in our assays (2:1) approximates the actual ratio of PMNs to mononuclear cells in blood, and the concentrations of 2B1 needed to overcome blocking of targeted lysis (100–1000 ng/ml) could be achieved at i.v. doses of BsMAb falling within the range of doses used in a variety of clinical studies of other monoclonal antibodies.

When c-erbB-2-positive tumor cells and CD16-positive effector cells are in juxtaposition, 2B1 BsMAb is clearly capable of causing cell complexing and cytosis. It is not known whether binding of 2B1 to CD16-positive effectors or PMNs will have other effects on cell activation or trafficking patterns before tumor cells are encountered. Binding of bivalent 3G8 to CD16 has been shown to initiate signal transduction in NK cells (60) and in monocytes (54). Heteroconjugates of bivalent 3G8 would presumably have effects at least as great. It remains to be seen in future experiments whether similar responses are mediated by the binding of 2B1 BsMAb, which possesses only a

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single 3G8-binding site but also possesses an Fc region capable (in principle) of interacting with CD16 or other Fcγ receptors. While most of our experiments were performed using effector cells from healthy human donors, we also sought preliminary evidence whether 2B1 could mediate targeted cytotoxicity using effector cells from cancer patients. 2B1 was able to mediate specific lysis against SK-BR-3 cells using effectors from each of three patients tested. When we used patient ascitic tumor cells as targets, 2B1 was also able to mediate lysis of these autologous tumor cells, although the levels of lysis observed were lower than for SK-BR-3 target cells. The lower lysis may correlate with our observation that c-erbB-2 expression on the ascitic tumor cells of this patient appeared to be substantially lower than expression on SK-BR-3 cells.

The competence of patient effector cells and the access of BsMAb and effector cells to tumor in vivo remain important issues for future exploration. Evaluation of 2B1 BsMAb in an SCID mouse/human tumor explant model will be reported elsewhere.4 Human antibody response to a murine/murine bispecific such as 2B1 is to be expected and would limit the duration of possible dosing in clinical trials. However, it is likely that such a response can be overcome or minimized by humanizing the 520C9- and 3G8-binding sites via cdr grafting. We believe such a response can be overcome or minimized by humanizing the 520C9- and 3G8-binding sites via cdr grafting. We believe such a response can be overcome or minimized by humanizing the 520C9- and 3G8-binding sites via cdr grafting.

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