Cancer Immunol Immunother (1995) 40: 182–190

ORIGINAL ARTICLE

M. Belen Moreno · Julie A. Titus · Michael S. Cole
J. Yun Tso · Nhat Le · Chang H. Paik · Tibor Bakács
Charles M. Zacharchuk · David M. Segal · John R. Wunderlich

Bispecific antibodies retarget murine T cell cytotoxicity against syngeneic breast cancer in vitro and in vivo

Received: 9 August 1994 / Accepted: 22 November 1994

Abstract Bispecific antibodies with specificity for CD3 and a tumor antigen can redirect cytolytic T cells to kill tumor targets, regardless of their natural specificity. To assess the clinical potential of bispecific antibodies for treatment of human cancers we have, in the present study, adapted a totally syngeneic mouse model to the targeting of mouse T cells against mouse tumors in immunocompetent mice. We show that gp52 of the mouse mammary tumor virus (MTV) can serve as a tumor-specific antigen for redirected cellular cytotoxicity. Chemically crosslinked and genetically engineered bispecific antibodies with specificities for gp52 and murine CD3 ε-chain induced activated mouse T cells to specifically lyse mouse mammary tumor cells from cultured lines and primary tumors from C3H-MTV$^+$ mice. Retargeted T cells also blocked the growth of mammary tumors in vitro as well as their growth in syngeneic mice. These findings identify murine MTV-induced mammary adenocarcinomas as a solid-tumor, animal model for retargeting T cells with bispecific antibodies against syngeneic breast cancer.

Key words Bispecific antibody · Redirected lysis
Targeted cytotoxicity · Mammary tumor
Mouse model

M. Belen Moreno · J. A. Titus · T. Bakács · D. M. Segal · J. R. Wunderlich
Experimental Immunology Branch, The National Cancer Institute, Bethesda, MD 20892, USA

M. Belen Moreno · C. M. Zacharchuk
Laboratory of Immune Cell Biology, The National Cancer Institute, Bethesda, MD 20892, USA

M. S. Cole · J. Yun Tso
Protein Design Labs Inc., Mountain View, CA 940431, USA

N. Le · C. H. Paik
Department of Nuclear Medicine, Clinical Center, NIH, Bethesda, MD 20892, USA

D.M. Segal (✉)
Building 10, Room 4B17, NIH, Bethesda, MD 20892-1360, USA

Introduction

T lymphocytes have been successfully retargeted with bispecific antibodies (bsAb) to react against selected tumors both in vitro and in animals [12, 33]. These bifunctional reagents react with triggering sites on the cytotoxic T cells and with cell-surface components on the tumor cells. Irrespective of their native specificities, the T cells are redirected by the bsAb to attack the selected tumor cells in a fashion that is independent of major histocompatability antigens.

A variety of animal models have been developed to explore the clinical potential of bsAb for use in treating cancer patients [4]. Several models have used targeted human T cells in immunocompromised mice to treat established human tumor xenografts, including ovarian carcinoma [13, 19], colon carcinoma [22, 23], and Hodgkin's lymphoma [27]. Although experiments using these models showed that targeted T cells could block tumor growth in vivo, their use in preclinical studies is limited. For example, xeno-responses between effector and host cells could give misleading results regarding the activation conditions needed to produce antitumor activity, or incompatabilities between donor and host adhesion molecules and their ligands might lead to abnormal migration of effector cells. A completely syngeneic mouse model in which a bsAb specifically targeted mouse T cells against mouse tumors would circumvent these problems. However, very few mAb are available that recognize tumor-specific or tumor-associated antigens in the mouse. Previous studies have used bsAb directed against immunoglobulin idiotypes expressed by B lymphomas [6, 42, 43] or against a Thy-1 alloantigen on a T-cell leukemia [38] to study targeted T cell responses against tumors in mice. However, with the exception of one study with a rat colon cancer cell line [5], T cells have not been targeted against solid tumors in syngeneic animal models, a challenging test for bsAb therapy.

The purpose of the present study was to retarget T cells to react against breast cancer cells in a completely syngeneic animal model. Toward this end, we have used mouse tumors induced by the mammary tumor virus (MTV) [18, 21, 30]. MTV is a B-type retrovirus that lacks an oncogene but nevertheless induces tumors by insertional mutagenesis [20]. After a lag period of 6–9 months following viral infection, mammary adenocarcinomas develop in most females of the highly susceptible mouse strains. The viral envelope protein, gp52, is expressed on the surface of mammary tumor cells induced by MTV [10, 20] and was chosen as the tumor marker for retargeting T cells with bsAb against cultured mammary tumor lines and primary tumor cells as well.

Several potential problems have limited the clinical development of bsAb. The most commonly used procedures for producing bsAb, chemical crosslinking or isolating the products of quadroma cell lines [12, 35, 36], have been troubled by inherent problems associated with complex chemical manipulations, with involved purification procedures, by variability between batches, and by instability of fused cell lines. In addition, the presence of multimers, aggregates, or intact Fc portions within bsAb preparations could lead to T cell receptor crosslinking in vivo, which has led to systemic T cell activation and dose-limiting toxicity in some clinical studies [1, 7, 9, 39]. For these reasons, we have tested a relatively new type of bsAb, a genetically engineered bispecific $F(ab')_2$ heterodimer [$bsF(ab')_2$] formed by using the known heteroreactivity of c-Fos and c-Jun leucine zippers [16]. Such $bsF(ab')_2$ proteins are relatively easy to produce and consist of well-defined, homogeneous, monomeric proteins that should be unable to crosslink T cell receptors in the absence of tumor cells. In the current study, we show that gp52-expressing mouse breast cancer cells are susceptible to cytotoxic T cells retargeted by either chemically crosslinked or genetically engineered bsAb both in standard in vitro assays and in syngeneic animals.

Materials and methods

Mice

C3H/HeNCrMTV$^-$, C57BL/6N, and BALB/cAnN (BALB/c) mice were obtained from NCI FCRDC (Frederick, Md.). They were used between 5 and 24 weeks of age. C3H/HeNCr MTV$^+$ exbreeder female mice with primary mammary tumors were obtained from the same source.

Tissue culture media

The "standard medium" used for culturing cells consisted of RPMI-1640 medium (Biofluids, Rockville, Md.) supplemented with 5% heat-inactivated fetal calf serum (FCS; Gibco BRL, Gaithersburg, Md.), glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 U/ml). "Activation medium" consisted of RPMI-1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 U/ml), glutamine (2 mM), pyruvate (1 mM), non-essential amino acids (1×) (M.A. Bioproducts; Walkersville, Md.), 50 μM 2-mercaptoethanol (Eastman Kodak, Rochester, N.Y.), and 10% heat-inactivated FCS. Sp2/0 transfectants expressing anti-CD3-Fos $F(ab')_2$ or anti-gp52-Jun $F(ab')_2$ were grown in serum-free medium consisting of a 1:1 mix of Iscove's modified Dulbecco medium/nutrient F12 supplemented with insulin (10 μg/ml) and transferrin (10 μg/ml).

Tumor cells

The following tumor cells were used: 64PT, a BALB/c MTV$^+$ tumor line generously provided by Dr. Fred Miller of the Michigan Cancer Foundation (Detroit, Mich.) [26]; C57BL/6 MC1 (B6MC1), a tumor that we induced with methylcholanthrene and adapted to growth in tissue culture; Mm5mt/c, an MTV$^+$C3H mammary adenocarcinoma line [24]; EL4, a C57BL/6N T-lymphoma line (available from ATCC, Rockville, Md.); and freshly explanted tumor cells from primary mammary tumors in C3HMTV$^+$ exbreeders. The cell lines were grown in standard medium. Mm5mt/c and 64PT cells were harvested following treatment with a mixture of 0.05% trypsin and 0.02% versine (Gibco BRL). B6MC1 cells grew as loosely adherent cells and were harvested by repeated pipetting. Cells from primary solid tumors were dispersed by a 2 to 3-h incubation of tissue slices in a combination of collagenase (type IV, Sigma, St. Louis, Mo.), hyaluronidase (Sigma type V), and DNase (Sigma, type IV) [29]. In some of the experiments tumor cells were surface-labeled with trinitrophenol [15].

Antibodies

The following polyclonal antibodies, mAb, and bsAb were used: P2AE12 (anti-gp52), an IgG2b mouse antibody against MTV gp52 [17] kindly provided as the hybridoma line by Dr. Larry Arthur (NCI FCRDC); 145-2C11 (2C11) (anti-CD3), a hamster IgG antibody against the ε chain of murine CD3 [14]; anti-NK1.1 mAb [37]; 2.4G2, an anti-(mouse Fc_γRII) mAb [41]; affinity-purified rabbit anti-dinitrophenol(DNP) antibody [32]; 2C11×P2AE12 (anti-CD3-Fos × anti-gp52-Jun) and 2C11 × anti-TAC (anti-CD3-Fos × anti-CD25-Jun), $bsF(ab')_2$ constructs, heterodimerized by c-Fos and c-Jun leucine zippers [16]. The V_L and V_H cDNA, used in the production and expression of P2AE12-F(ab')-Jun were cloned as described [16]; 2C11 × P2AE12 [anti-CD3 × anti-gp52 (heteroconjugate)] and 2C11 × anti-DNP [anti-CD3 × anti-DNP (heteroconjugate)], produced by chemically crosslinking intact mAb [40]. All mAb were purified from ascites fluid by ion-exchange chromatography and gel filtration [2]. $bsF(ab')_2$ proteins were purified by ion-exchange chromatography [16].

Flow cytometry

Cells (10^6/tube) were incubated with saturating concentrations of fluorescein-isothiocyanate(FITC)-labeled anti-gp52 mAb in the presence of anti-Fc_γR mAb and washed as described [34]. Fluorescence was measured on viable cells using either a FACStar$^+$ or a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.) with 488-nm argon laser excitation. Dead cells were excluded on the basis of low- and high-angle light scatter or on their labeling with propidium iodide.

Radiolabeled-antibody-binding assays

Antibody binding was determined essentially as described by Dower et al. [11]. Briefly, antibodies were radiolabeled by the chloramine T method to a specific activity of 3–5 μCi/μg. Binding assays were

done at 0° C in Hanks balanced salts solution (HBSS) containing 0.1% bovine serum albumin. Cells (160 µl at 3×10^7/ml), serial twofold dilutions of radiolabeled antibody (32 µl), and medium (160 µl) were mixed in 1.5-ml Eppendorf centrifuge tubes and incubated for 3 h on ice. In control samples, medium was replaced by 160 µl unlabeled antibody at 1 mg/ml. After mixing, 110 µl aliquots of the suspensions were layered on 200 µl FCS in 400-µl microcentrifuge tubes, in triplicate, and centrifuged at 14000 rpm for 20 sec. The bottoms of the tubes were cut and measured in a gamma counter. The number of molecules bound per cell was calculated from the difference between experimental and control values, using the specific activities of the antibodies, and the numbers of cells used. The number of binding sites and the affinity constant were determined by Scatchard analysis. Affinity constants were also estimated from the amount of unlabeled antibody giving 50% inhibition of binding of a constant amount of radiolabeled antibody. Radiolabeled bs$(Fab')_2$ was stable for at least 48 h at 37° C in serum by HPLC size analysis.

Effector cells used in cytolysis experiments

For in vitro cytolysis experiments, splenocytes from C57BL/6 mice were used as effectors because they could be depleted of natural killer (NK) activity; they were NK-depleted by incubating them for 40 min at 0° C with an optimal concentration of anti-NK1.1 in HBSS containing 5% FCS. They were then suspended in a 1/10 dilution of Low-Tox-M rabbit complement (Cedarlane, Hornby, Ontario), incubated for 40 min at 37° C. These cells were cultured in activation medium containing 100 Cetus units/ml human recombinant interleukin-2 (rIL-2; Chiron, Emeryville, Calif.) for 5 days by two procedures: In the experiments shown in Fig. 2, cells were incubated in V-bottom microtiter wells at $(0.5–1) \times 10^6$ cells/ml, 200 µl/well. For Fig. 3, cells were incubated in 25-cm^2 flasks, 2×10^6 cells/ml, 10 ml/flask. On day 3, 5 ml activation medium was added to each flask.

Effector cells in growth-inhibition experiments

Dispersed splenocytes from BALB/c mice were cultured for 5 days in activation medium containing irradiated (2000 cGy) C57BL/6 splenocytes (one-half the number of BALB/c cells), rIL-2 (10 Cetus units/ml), chromatographically purified bacterial lipopolysaccaride from wild-type *S. minnesota* (50 ng/ml) (List Biological Labs., Campbell, Calif.), and 1 µM indomethacin (Sigma). Splenocytes were cultured in either 10 ml medium in horizontal 25-cm^2 tissue-culture flasks (2×10^7 cells/flask) for in vitro growth-inhibition assays, or in 25 ml medium in horizontal 75-cm^2 tissue-culture flasks (7×10^7 cells/flask) for in vivo assays. On day 3, for the in vitro growth inhibition assays, an additional one-half volume of fresh activation medium was added to each flask. This method of activation was chosen because it gave consistent anti-tumor activity in vivo.

In vitro cytolysis assays

bsAb and anti-Fc_γR mAb were added to effector cells and both were serially diluted; this was followed by addition of $(5–10) \times 10^3$ ^{51}Cr-labeled target cells. After 3–4 h incubation, lysis was determined by ^{51}Cr release as described [15]. Data points represent the averages of three or four replicates and standard errors were always within 10% of the mean.

In vitro growth-inhibition assay

The ability of retargeted T cells to inhibit the growth of tumor cells in vitro was measured using a modification of the procedure of Qian et al. [25]. Graded numbers of activated BALB/c spleen cells were cultured in round-bottom microtiter wells with bsAb and 5×10^3 64PT cells (which grow adherently) for 3 days in a total volume of 0.2 ml in standard medium containing 10 units/ml rIL-2. Non-adherent cells were removed by washing three times and the number of remaining viable adherent cells was determined by adding 100 µl standard medium to each well, followed by 25 µl 1 mg/ml 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt (XTT) [28] (Diagnostic Chemical, Charlottetown, P.E.I., Canada). Plates were incubated for 2–4 h at 37° C, and the A_{450} determined in an enzyme-linked immunosorbent assay reader. For some experiments, results are expressed as units of growth inhibition, analogous to the lytic unit used in cytolysis assays [8]. One unit of growth inhibition equals 10^6 divided by the number of effector cells causing 50% inhibition of the maximum growth.

In vivo tumor neutralization assay

Tumor neutralization tests were carried out by using a modification of the procedure described by Winn [44]. Normal adult female BALB/c mice, five per group, were injected subcutaneously on day 0 in the flank with 0.1 ml phosphate-buffered saline containing 1×10^6 64PT cells, activated BALB/c splenocytes, variable amounts of bsAb and 83 µg/ml anti-Fc_γR mAb. Preliminary experiments showed that 10^6 64Pt cells injected alone grew in all mice tested, while 10^5 cells grew in four out of five mice, and 5×10^4 cells grew in only two of five mice. Effector cells and antibody were incubated on ice for at least 30 min and were then mixed with tumor cells at room temperature immediately before injection. Tumor size was measured as the average of two perpendicular diameters for 1–2 months, and mice were sacrificed when tumors exceeded 2 cm in average diameter. Data are reported in terms of the numbers of mice that either had or had not developed palpable tumor by the end of the experiment. Tumor-positive mice always had tumors greater than 1 cm in average diameter.

Results

The expression of gp52 by MTV-induced mammary tumor cells

The relative levels of gp52 expressed by cells from the 64PT and Mm5mt/c MTV-induced tumor cell lines and by B6MC1 control cells are shown in Fig. 1A–C. The 64PT and Mm5mt cells gave brightly-labeled single peaks with FITC-anti-gp52, whereas the B6MC1 cells were negative. Since C3H MTV$^+$ mice develop primary mammary tumors with high frequency, we examined several of these tumors from ex-breeder female mice for gp52 expression. Fluorescence-activated cell sorter (FACS) profiles of single-cell suspensions prepared from three representative freshly explanted tumors from different mice are shown in Fig. 1D–F. Cells from the primary tumors showed greater heterogeneity in gp52 staining than the cultured tumor lines, but the contribution of non-tumor stromal cells to the gp52 profiles is unknown.

The binding of P2AE12 to 64PT cells was measured quantitatively using a radioactive binding assay. As seen from the data presented in Table 1, the intact P2AE12 binds with an affinity (K_a) of about 10^8 M^{-1},

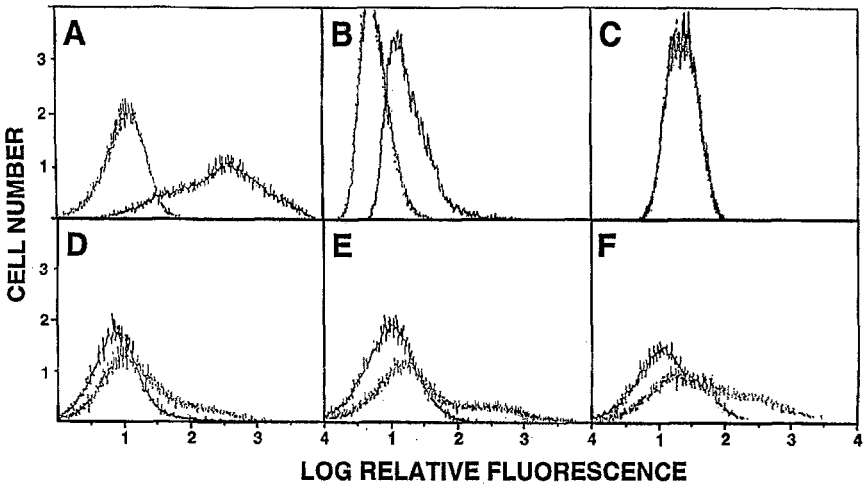

Fig. 1A–F gp52 expression by various cells. **A** 64PT mammary tumor line; **B** Mm5mt mammary tumor line; **C** B6MC1 methylcholanthrene-induced tumor line; **D–F** freshly explanted C3H primary mammary adenocarcinoma cells from different mice. Cells were labeled with fluorescein-isothiocyanate (FITC)-anti-gp52 or, as a control, with FITC-anti-Leu4, and analyzed by flow cytometry

Table 1 Binding constants and numbers of binding sites for parental and bispecific antibodies. K_i values were determined as the reciprocal of the concentration giving 50% inhibition of binding of radiolabeled intact parental antibody. K_a values were determined by Scatchard analyses of direct binding curves. *ND* not determined

Antibody	Cell	$10^{-7} \times K_i$ (M^{-1})	$10^{-7} \times K_a$ (M^{-1})	$10^{-4} \times N$ (molecules/cell)
2C11	EL4	10.0	9.8[a]	1.9[a]
2C11 Fab	EL4	0.67	ND	ND
P2AE12	64PT	8.3	11.0[a]	8.2[a]
P2AE12 Fab	64PT	0.14	ND	ND
2C11 × P2AE12 [bsF(ab′)$_2$]	EL4	1.0	3.6	1.7
	64PT	0.29	ND	ND

[a] The average of two independent determinations

as judged by Scatchard analysis of the binding curves or by inhibition of binding of radiolabeled P2AE12 with unlabeled antibody. Approximately 8×10^4 antibody molecules bind per cell at saturation, suggesting that each 64PT cell expresses, on average, 1.6×10^5 gp52 molecules, assuming that each antibody binds divalently. The P2AE12 Fab binds with a much lower affinity than the intact antibody, and was estimated by inhibition experiments to bind with a K_a of about $(1–2) \times 10^6$ M^{-1}.

Cytolysis of MTV-induced tumor cells by retargeted T cells

We tested whether the chemically crosslinked intact bispecific antibody, anti-CD3 × anti-gp52 (heteroconjugate), could induce splenic T cells to lyse gp52$^+$ tumor cells. Activated C57BL/6 spleen cells, which were allogeneic to some targets, were used as effectors because NK cells could specifically be removed from splenocytes of this strain (see Materials and methods). Such effector cells, targeted with either anti-CD3 × anti-gp52 (heteroconjugate) or anti-CD3 × anti-DNP (heteroconjugate) bsAb mediated the lysis of trinitrophenol-modified Mm5mt/c MTV-induced tumor cells (Fig. 2A), but a mixture of intact anti-CD3 plus anti-gp52 was unable to mediate lysis. In other experiments (not shown) we found that bsAb in the absence of effector cells were not toxic for tumor cells and splenic effector cells that were freshly explanted or precultured without activating agents were unable to mediate targeted lysis. The specificity of lysis is shown in Fig. 2B, where trinitrophenol-modified B6MC1 cells (which do not express gp52, Fig. 1) are lysed by effector cells targeted with anti-CD3 × anti-DNP (heteroconjugate), but not with anti-CD3 × anti-gp52 (heteroconjugate). Freshly explanted primary mammary adenocarcinoma cells were also lysed by the cytolytic T cells targeted with anti-CD3 × anti-gp52 (heteroconjugate) (Fig. 2C).

For in vivo studies, it would be preferable to use a bsAb with a well-defined chemical structure and size and without an Fc portion. We therefore produced a genetically engineered bsF(ab′)$_2$ from the 2C11 anti-CD3 mAb and the P2AE12 anti-gp52 mAb, using a leucine zipper to direct the formation of the heterodimeric structure. Table 1 shows that this bsAb bound to both CD3 (EL4 cells) and gp52 (64PT cells) with similar, but slightly higher affinities than the Fab fragments of the parental antibodies. Since the K_i values are only approximate estimates of binding constants, it is not clear that the small differences in affinities between the Fab and the bsAb are significant. Figure 3 compares the lysis mediated by the genetically engineered bsF(ab′)$_2$ with that mediated by the conventional heteroconjugate. Despite the low affinity for both effector and target cells, the bsF(ab′)$_2$ successfully retargeted lysis against 64PT tumor cells. In fact, the cytolytic activity induced by anti-CD3 × anti-gp52 F(ab′)$_2$ was typically higher than that from the

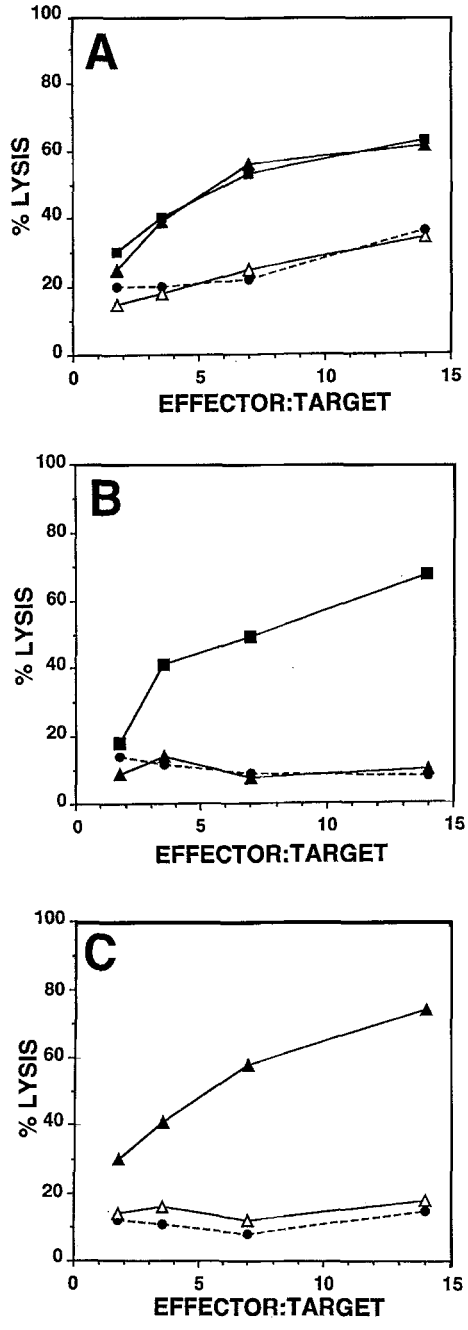

Fig. 2A–C Lysis of $gp52^+$ tumor cells by retargeted cytolytic T cells. Preactivated C57BL/6 spleen cells were retargeted with (■) anti-CD3 × anti-dinitrophenol(DNP) heteroconjugate, 2 μg/ml; (▲) anti-CD3 × anti-gp52 heteroconjugate, 2 μg/ml; (△) a mixture of anti-CD3 plus anti-gp52, 1 μg/ml each, and (●) no antibody, and tested for the ability to lyse **A** trinitrophenol(TNP)-labeled Mm5mt/c cells ($gp52^+$); **B** TNP-labeled B6MC1 cells ($gp52^-$), and **C** freshly explanted primary mammary adenocarcinoma cells ($gp52^+$). All wells contained 2 μg/ml 2.4G2 anti-$Fc_\gamma R$ mAb. Levels of spontaneous lysis ranged from 8% for TNP-modified 64PT cells to 20% for freshly explanted tumor cells

randomly crosslinked bsAb. Intact anti-CD3 × anti-DNP heteroconjugate failed to mediate killing of 64PT.

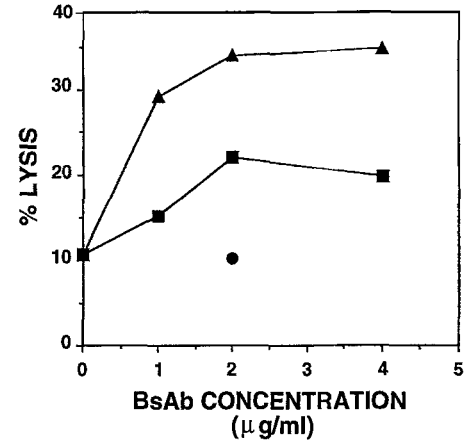

Fig. 3 Comparison of lysis induced by randomly crosslinked (■) and genetically engineered (▲) anti-CD3 × anti-gp52 bsAb. Preactivated, natural-killer-depleted C57BL/6 effector spleen cells were incubated with 64PT target cells at an effector : target ratio of 25 : 1. Anti-CD3 × anti-DNP heteroconjugate (●) was used as a negative control

Inhibition of in vitro growth of mammary tumor cells by targeted T cells

Earlier studies of T cells retargeted against tumor cells with heteroconjugates showed a closer correlation of in vivo antitumor activity with in vitro growth inhibition than with in vitro cytolysis of the tumor cells [25]. Thus, we tested whether murine T cells could inhibit, in vitro, the growth of a syngeneic MTV^+ mammary tumor line when retargeted against gp52 with bsAb. To this end, alloactivated BALB/c splenocytes were co-incubated for 3 days with anti-CD3 × anti-gp52 bsAb and 64PT, after which nonadherent cells were removed and the number of remaining viable tumor cells determined colorimetrically using the XTT assay [28].

Splenic T cells, retargeted by either anti-CD3 × anti-gp52 heteroconjugate or by the $bsF(ab')_2$, inhibited tumor growth. Figure 4 shows a dose response curve for the anti-CD3 × anti-gp52 $bsF(ab')_2$ and illustrates how the growth inhibition units presented in Table 2 were calculated. These units, like lytic units [8], are used to compare antitumor activities between treatment groups within an experiment. Intact anti-CD3, anti-CD3 × anti-DNP (heteroconjugate), and anti-CD3 × anti-CD25 $bsF(ab')_2$, were much less effective in blocking tumor growth when compared to the anti-CD3 × anti-gp52 bsAb (Table 2). What is more important, the addition of antibodies to the $Fc_\gamma R$ reduced the potency of the randomly crosslinked bsAb and anti-CD3 but did not interfere with the cytotoxic activity of the anti-CD3 × anti-gp52 $bsF(ab')_2$. The activity of intact 2C11 alone in inhibiting tumor growth is consistant with earlier studies [3] showing that human peripheral blood T cells mediate tumor growth inhibition by secreting cytotoxic or cytostatic factors upon

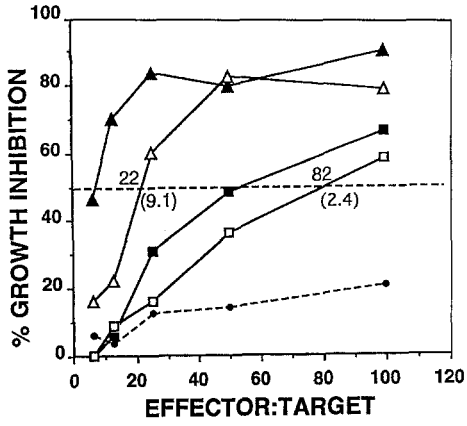

Fig. 4 Inhibition of growth of $gp52^+$ tumor cells in vitro by retargeted T cells. Activated spleen cells were cultured with 5×10^3 64PT mammary adenocarcinoma cells/well for 3 days with the following concentrations of anti-CD3 × anti-gp52 bsF(ab′)$_2$: ▲ 3 μg/ml, △ 0.75 μg/ml, ■ 0.2 μg/ml, □ 0.05 μg/ml, ● no antibody. The number of adherent tumor cells was determinated on day 3 and the percentage inhibition of tumor growth relative to tumor alone was calculated. X-axis values refer to ratio of splenocytes to tumor cells on day 0. The growth inhibition units were calculated from the effector:target ratios giving 50% inhibition of tumor growth, as described in Materials and methods. As examples, the effector:target ratios at the 50% inhibition points (intersections of experimental curves with dashed lines) are indicated for curves representing 0.05 μg/ml and 0.75 μg/ml bsAb, and the calculated growth inhibition units are shown in parentheses below the dashed (50% inhibition) line. This figure shows how the growth inhibition units presented in Table 2 were calculated

Table 2 In vitro inhibition of 64PT tumor cell growth by activated murine T cells targeted with bispecific antibodies. Growth inhibition units (*u*) were calculated from the effector:target ratios giving 50% inhibition of tumor growth on day 3, as described in Materials and methods. Effector cells were BALB/c splenocytes preactivated for 5 days. Where indicated, 1 μg/ml 2.4G2 anti-$Fc_\gamma R$ antibody was added. $Fc_\gamma R$ Fc_γ receptor, *DNP* dinitrophenol

Antibody	Antibody (μg/ml)	Growth inhibition	
		−anti-$Fc_\gamma R$	+anti-$Fc_\gamma R$
None	–	<2	<2
Anti-CD3	3	13.8	2.9
Anti-CD3 × anti-gp52 (heteroconjugate)	3	<31	25.3
Anti-CD3 × anti-DNP (heteroconjugate)	3	6.9	2.9
Anti-CD3 × anti-gp52 [bsF(ab′)$_2$]	3	27.8	28.6
	0.75	9.1	–
	0.19	3.6	–
	0.05	2.4	–
Anti-CD3 × anti-CD25 [bsF(ab′)$_2$]	3	2.6	3.0

T cell receptor (TcR) crosslinking. The decreased activity of 2C11 mAb in the presence of anti-$Fc_\gamma R$ antibody suggests that $Fc_\gamma R^+$ cells crosslink TcR and activate T cells when intact antibodies to CD3 are present. The source of the low, but significant, amount of tumor growth inhibition observed with anti-CD3 × anti-(human)CD25 bsF(ab′)$_2$ is unknown. It is possible that a small amount of aggregates in the preparation caused this non-specific inhibition.

Neutralization of tumor growth in vivo

Lastly, preactivated, retargeted T cells were able to block the growth of syngeneic mammary tumor cells in vivo. We chose the 64PT tumor for these experiments, because the tumor cells grew rapidly and reliably when injected subcutaneously into normal syngeneic hosts. Data presented in Table 3 show that the growth of 64PT tumor cells was totally blocked by preactivated splenocytes retargeted with anti-CD3 × anti-gp52 bsF(ab′)$_2$ and anti-CD3 × anti-gp52 (heteroconjugate), even in the presence of anti-$Fc_\gamma R$ mAb. As a control, the non-gp52-binding bsAb, anti-CD3 × anti-CD25 bsF(ab′)$_2$, failed to prevent tumor growth, showing both the requirement for tumor specificity and the inability of anti-CD3 in this irrelevant bsF(ab′)$_2$ to interfere with tumor growth. Intact anti-CD3 or mixtures of intact anti-CD3 and anti-gp52 partially blocked tumor growth in some experiments but not in others (data not shown). It is likely that this activity is due to the release of cytokines induced by TcR crosslinking as seen in the in vitro experiments of Table 2. Anti-CD3 × anti-gp52 [heteroconjugate or bsF(ab′)$_2$] failed to block tumor growth in the absence of effectors (data not shown).

Discussion

In the present study we demonstrate that mouse mammary tumors growing in normal mouse strains treated with a bsAb having anti-CD3 and anti-gp52 specificities provide a suitable model for testing the clinical potential of bsAb therapy. We and others [13, 19, 22, 23, 27] have previously shown that targeted human T cells could block the growth of human tumor xenografts in immunocompromised mice, but these studies could not adequately address problems related to T cell activation and lymphocyte homing. In searching for a syngeneic animal model our greatest problem was to find a tumor-specific antigen that could be recognized by the target-specific half of the bsAb. The mammary adenocarcinomas used in this study express gp52, which, we show, allows tumor-specific retargeting. gp52 is an envelope glycoprotein expressed by the MTV that induced the tumors. MTV acquires its envelope from the host-cell plasma membrane during a budding process that does not lyse the tumor cell, and gp52 is expressed on the tumor-cell surface in the absence of mature virus production [31]. The mammary adenocarcinoma line, 64PT, expresses relatively high

Table 3 Inhibition of subcutaneous growth of 64PT tumors in BALB/c mice. Tumor cells (10^7/ml) and activated BALB/c splenocytes were mixed with the indicated bispecific antibodies and 2.4G2 anti-$Fc_\gamma R$ mAb (83 μg/ml) at an effector : target ratio of 10 : 1, and 0.1 ml was injected per mouse on day 0. Data indicate the number of mice with ($tumor^+$) and without ($tumor^-$) detectable subcutaneous tumors on day 42 (experiment 1) or day 56 (experiment 2)

Effector cells	Antibody	Antibody per mouse (μg)	$Tumor^+$ mice	$Tumor^-$ mice
Experiment 1				
–	None	–	5	0
+	None	–	5	0
+	Anti-CD3 × anti-gp52 [$bsF(ab')_2$]	8.3	0	5
+	Anti-CD3 × anti-gp52 [$bsF(ab')_2$]	4.1	0	5
+	Anti-CD3 × anti-gp52 (heteroconjugate)	8.3	0	5
Experiment 2				
–	None	–	5	0
+	None	–	5	0
+	Anti-CD3 × anti-gp52 [$bsF(ab')_2$]	10	0	5
+	Anti-CD3 × anti-gp52 [$bsF(ab')_2$]	3.3	1	4
+	Anti-CD3 × anti-gp52[$bsF(ab')_2$]	1.1	0	5
+	Anti-CD3 × anti-CD25 [$bsF(ab')_2$]	10	5	0
+		5	5	0

amounts of gp52 (Fig. 1), and grows well when transplanted subcutaneously in BALB/c mice (Table 3). We do not know whether 64PT sheds MTV particles but, if it does, the levels of shed virus were too low to block targeted antitumor activity in our studies. Among the mouse strains that develop MTV-induced breast cancer, gp52 is selectively expressed by the mammary adenocarcinoma cells, and our studies show that tumor cells dispersed from primary MTV-induced tumors can be lysed in vitro by retargeted T cells (Fig. 2).

Mouse T cells, retargeted with an appropriate bsAb, generate antitumor activity against mouse mammary adenocarcinoma cells in each of three different types of assay: cytolysis (Figs. 2, 3), growth inhibition in vitro (Table 2) and tumor neutralization in mice (Table 3). Cytolysis and growth inhibition are generally thought of as representing two fundamentally different mechanisms. Lysis, as measured in short-term ^{51}Cr-release assays, requires a direct link between effector and target cell, and involves the exocytosis of cytolytic components at the effector cell : target cell interface. By contrast, growth inhibition, as measured by longer-term viability assays, can be mediated by cytokines, such as tumor necrosis factor α or interferon γ, that are secreted into the medium and act directly on tumor cells, regardless of whether they are in close contact with the effector lymphocytes [3, 25]. Both cytolysis and growth inhibition require TcR crosslinking, but cytokine secretion can occur with TcR crosslinking in the absence of tumor targets. This mechanism could account for the observation in Table 2 that intact anti-CD3 can inhibit tumor growth in vitro, a blockage that is reversed by anti-$Fc_\gamma R$ mAb presumably by preventing TcR crosslinking. In addition to directly acting on tumors, cytokines released in vivo at a tumor site could potentially create an inflammatory response, attracting and activating other types of effectors, such as macrophages, NK cells, and granulocytes.

The existence of mAb against murine CD3 and gp52 has made possible the production of bsAb that specifically target T cells against mammary tumors. We compared the targeting ability of a genetically engineered $bsF(ab')_2$ with that of a conventional randomly crosslinked bsAb, and found the $bsF(ab')_2$ to be a superior targeting agent in mediating lysis (Fig. 3), and roughly equivalent to the random crosslink in mediating tumor growth inhibition in vitro (Table 2) and tumor neutralization in vivo (Table 3). Although the heteroconjugates are easier to produce than the genetically engineered $bsF(ab')_2$ proteins, they exhibit batch-to-batch variation in aggregation state and chemical composition. These bsAb would therefore be expected to give variable results in animals and would be unsuitable for studies done in patients. Compared to other homogeneous bsAb preparations such as quadromas or hinge-disulfide-linked hetero-$F(ab')_2$, the Fos-Jun-linked $bsF(ab')_2$ proteins should be much easier to produce and isolate in pure form. Moreover, the absence of the Fc region on the genetically engineered $bsF(ab')_2$ proteins removes the need to digest the product with proteases. Removal of the Fc piece will probably be essential for in vivo targeting to prevent the removal, lysis, or premature activation of T cells by $Fc_\gamma R$-bearing cells. Premature activation is a real concern, since general activation of T cells in humans by antibodies that bind to the TcR has been associated with central nervous system toxicity [1, 7, 9], and similar toxicity has been reported with bsAb [39].

Our next challenge is to treat established tumors in vivo. Initially we will examine the homing, biodistribution, and pharmacokinetics of injected bsAb. Because the $bsF(ab')_2$ binds monovalently to both tumor and T cell, it interacts weakly with both. This may make it difficult to detect the homing of $bsF(ab')_2$ to T cells and tumor, but may not adversely affect redirected tumor cytotoxicity, where multivalent interactions between

effector and targets would increase the overall avidity of the interaction between effector and target cells. Transplantable tumors such as 64PT will be used first, but since more than 80% of female mice among susceptible MTV^+ strains develop mammary cancers by 1 year of age, we can also experiment with primary tumors. The development of these syngeneic animal models should test the true potential of bispecific antibody therapy and aid in its optimization for use in human cancer patients.

Acknowledgements We thank Dr. Gilbert Smith for valuable discussions about the MTV mouse model.

References

1. Agarwal RK, Ostaszewski ML, Feld LG, Springate JE, Moxey-Mims MM, O'Neil KM (1993) Tumor necrosis factor and interleukin-6 in cerebrospinal fluid of a patient with recurrent adverse central nervous system events following OKT3. Transplant Proc 25:2143
2. Andrew SM, Titus JA (1992) Purification and fragmentation of antibodies: In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W (eds) Current protocols in immunology, vol 1. Current Protocols, New York, p 2.7.1
3. Barth RJ, Jr., Mule JJ, Spiess PJ, Rosenberg SA (1991) Interferon gamma and tumor necrosis factor have a role in tumor regressions mediated by murine $CD8^+$ tumor-infiltrating lymphocytes. J Exp Med 173:647
4. Beun GD, Velde CJ van de, Fleuren GJ (1994) T-cell based cancer immunotherapy: direct or redirected tumor-cell recognition? Immunol Today 15:11
5. Beun GD, Velde CJ van de, Fleuren GJ, Eggermont AM (1993) T-cell retargeting using bispecific monoclonal antibodies in a rat colon carcinoma model: IV. Tumor neutralization in Winn type assays. J Immunother 14:11
6. Brissinck J, Demanet C, Moser M, Leo O, Thielemans K (1991) Treatment of mice bearing BCL1 lymphoma with bispecific antibodies. J Immunol 147:4019
7. Capone PM, Cohen ME (1991) Seizures and cerebritis associated with administration of OKT3. Pediatr Neurol 7:299
8. Cerottini J-C, Brunner KT (1974) Cell-mediated cytotoxicity, allograft rejection, and tumor immunity. Adv Immunol 18:67
9. Charpentier B, Hiesse C, Lantz O, Ferran C, Stephens S, O'Shaugnessy D, Bodmer M, Benoit G, Bach JF, Chatenoud L (1992) Evidence that antihuman tumor necrosis factor monoclonal antibody prevents OKT3-induced acute syndrome. Transplantation 54:997
10. Dickson C, Peters G (1983) Proteins encoded by mouse mammary tumour virus. Curr Top Microbiol Immunol 106:1
11. Dower SK, Ozato K, Segal DM (1984) The interaction of monoclonal antibodies with MHC class I antigens on mouse spleen cells. I. Analysis of the mechanism of binding. J Immunol 132:751
12. Fanger MW, Morganelli PM, Guyre PM (1992) Bispecific antibodies. Crit Rev Immunol 12:101
13. Garrido MA, Valdayo MJ, Winkler DF, Titus JA, Hecht TT, Perez P, Segal DM, Wunderlich JR (1990) Targeting human T-lymphocytes with bispecific antibodies to react against human ovarian carcinoma cells growing in nu/nu mice. Cancer Res 50:4227
14. Hoffman RW, Bluestone JA, Leo O, Shaw S (1985) Lysis of anti-T3-bearing murine hybridoma cells by human allospecific cytotoxic T cell clones and inhibition of that lysis by anti-T3 and anti-LFA-1 antibodies. J Immunol 135:5
15. Karpovsky B, Titus JA, Stephany DA, Segal DM (1984) Production of target-specific effector cells using hetero-cross-linked aggregates containing anti-target cell and anti-Fcγ receptor antibodies. J Exp Med 160:1686
16. Kostelny SA, Cole MS, Tso JY (1992) Formation of a bispecific antibody by the use of leucine zippers. J Immunol 148:1547
17. Massey RJ, Schochetman G (1981) Topographical analysis of viral epitopes using monoclonal antibodies: mechanism of virus neutralization. Virology 115:20
18. Medina D (1982) Mammary tumors: In: Foster HL, Small JD, Fox JG (eds) The Mouse in Biomedical Research. Academic Press, New York, p 373
19. Mezzanzanica D, Garrido MA, Neblock DS, Daddona PE, Andrew SM, Zurawski VR, Segal DM, Wunderlich JR (1991) Human T-lymphocytes targeted against an established ovarian carcinoma with bispecific $F(ab')_2$ antibody prolong host survival in a murine xenograft model. Cancer Res 51:5716
20. Michalides R, Ooyen A van, Nusse R (1983) Mouse mammary tumor virus expression and mammary tumor development. Curr Top Microbiol Immunol 106:57
21. Nandi S, McGrath CM (1973) Mammary neoplasia in mice. Adv Cancer Res 17:353
22. Nelson H, Ramsey PS, Kerr LA, McKean DJ, Donohue JH (1990) Regional and systemic distribution of anti-tumor × anti-CD3 heteroaggregate antibodies and cultured human peripheral blood lymphocytes in a human colon cancer xenograft. J Immunol 145:3507
23. Nelson H, Ramsey PS, McKean DJ, Dozois RR, Donohue JH (1991) Anti-tumor × anti-lymphocyte heteroconjugates augment colon tumor cell lysis in vitro and prevent tumor growth in vivo. Dis Colon Rectum 34:140
24. Owens RB, Hackett AJ (1972) Tissue culture studies of mouse mammary tumor cells and associated viruses. J Natl Cancer Inst 49:1321
25. Qian JH, Titus JA, Andrew SM, Mezzanzanica D, Garrido MA, Wunderlich JR, Segal DM (1991) Human peripheral blood lymphocytes targeted with bispecific antibodies release cytokines that are essential for inhibiting tumor growth. J Immunol 146:3250
26. Rak JW, McEachern D, Miller FR (1992) Sequential alteration of peanut agglutinin binding-glycoprotein expression during progression of murine mammary neoplasia. Br J Cancer 65:641
27. Renner C, Jung W, Sahin U, Denfeld R, Pohl C, Trumper L, Hartmann F, Diehl V, Van Lier R, Pfreundschuh M (1994) Cure of xenografted human tumors by bispecific monoclonal antibodies and human T cells. Science 264:833
28. Roehm NW, Rodgers GH, Hatfield SM, Glasebrook AL (1991) An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. J Immunol Methods 142:257
29. Rosenberg SA, Mule JJ, Spiess PJ, Reichert CM, Schwarz SL (1985) Regression of established pulmonary metastases and subcutaneous tumor mediated by the systemic administration of high-dose recombinant interleukin 2. J Exp Med 161:1169
30. Salmons B, Gunzburg WH (1987) Current perspectives in the biology of mouse mammary tumour virus. Virus Res 8:81
31. Sarkar NH, Racevskis J (1983) Expression and disposition of the murine mammary tumor virus (MuMTV) envelope gene products by murine mammary tumor cells. Virology 126:279
32. Segal DM, Hurwitz E (1976) Dimers and trimers of immunoglobulin G covalently cross-linked with a bivalent affinity label. Biochemistry 15:5253
33. Segal DM, Snider DP (1989) Targeting and activation of cytotoxic lymphocytes. Chem Immunol 47:179
34. Segal DM, Titus JA Stephany DA (1987) The use of fluorescence flow cytometry for the study of lymphoid cell receptors: In: Di Sabato G (ed) Methods of enzymology: immunological techniques, part K. In vitro models of B and T cell function and lymphoid cell receptors. Academic Press, New York, p 478

35. Segal DM, Urch CE, George AJT, Jost CR (1992) Bispecific antibodies in cancer treatment: In: DeVita VT, Hellman S, Rosenberg SA (eds) Biologic therapy of cancer updates, vol 2. Lippincott, Philadelphia, p 1
36. Segal DM, Jost CR, George AJT (1993) Targeted cellular cytotoxicity: In: Sitkovsky MV, Henkart PA (eds) Cytotoxic cells: generation, recognition, effector functions, methods. Springer, Berlin Heidelberg New York, p 96
37. Sentman CL, Hackett JJr, Moore TA, Tutt MM, Bennett M, Kumar V (1989) Pan natural killer cell monoclonal antibodies and their relationship to the NK1.1 antigen. Hybridoma 8:605
38. Staerz UD, Bevan MJ (1986) Use of anti-receptor antibodies to focus T cell activity. Immunol Today 7:241
39. Tibben JG, Boerman OC, Claessens RAMJ, Corstens FHM, Van Deuren M, De Mulder PHM, Van der Meer JWM, Keijser KGG, Massuger LFAG (1993) Cytokine release in an ovarian cancer patient following intravenous administration of bispecific antibody OC/TR F(ab')2. J Natl Cancer Inst 85:1003
40. Titus JA, Garrido MA, Hecht TT, Winkler DF, Wunderlich JR, Segal DM (1987) Human T cells targeted with anti-T3 cross-linked to antitumor antibody prevent tumor growth in nude mice. J Immunol 138:4018
41. Unkeless JC (1979) Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. J Exp Med 150:580
42. Weiner GJ, Hillstrom JR (1991) Bispecific anti-idiotype/anti-CD3 antibody therapy of murine B cell lymphoma. J Immunol 147:4035
43. Weiner GJ, Kostelny SA, Hillstrom JR, Cole MS, Link BK, Wang SL, Tso JY (1994) The role of T cell activation in anti-CD3 X antitumor bispecific antibody therapy. J Immunol 152:2385
44. Winn HJ (1961) Immune mechanisms in homotransplantation. II. Quantitative assay of the immunologic activity of lymphoid cells stimulated by tumor homografts. J Immunol 86:228