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Immunology

Universal bispecific antibody for targeting tumor cells for destruction by cytotoxic T cells

(bispecific monoclonal antibody/cytotoxic T lymphocyte/CD3 antibody/cytolysis)

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ABSTRACT **Previous studies have demonstrated that bispecific hybrid antibodies produced by cell–cell fusion or chemically conjugated heteroaggregates can direct cytotoxic T lymphocytes to kill target cells for which they have no intrinsic specificity, a phenomenon we call effector cell retargeting (ECR). These studies used bispecific reagents with one specificity directed to CD3 or Ti on the effector cell and the other directed to a target cell antigen. To avoid the need to create different hybrid hybridomas for each target antigen we have developed a universal means to elicit ECR through the use of an antiglobulin step. We have constructed a bispecific hybrid antibody with dual specificity for CD3 and a rat immunoglobulin light chain allotype. This bispecific antibody could mediate ECR to a range of target cells, each coated with distinct surface-binding rat monoclonal antibodies. A particular advantage of targeting to surface-bound monoclonal antibodies is that all other available effector systems may also attack the same antibody-coated target cell.**

Tumor serotherapy has concentrated on two main areas. The first has exploited the Fc regions of monoclonal antibodies to harness natural effector systems such as complement (1), antibody-dependent cell-mediated cytotoxicity (ADCC), and opsonization (2–4); the second has aimed to deliver covalently conjugated cytotoxic drugs, toxins, or radioisotopes to specific target sites (5). Recently, there has been increasing interest in harnessing the cytotoxic potential of T cells as well. The need to recognize a tumor antigen can be bypassed by cross-linking target cells to activated T cells using heteroconjugates (6–8) or bispecific antibodies (9–11) to the T-cell receptor complex (Ti and CD3) and to target cell-surface antigens. This process we call effector cell retargeting (ECR). *In vivo* bispecific antibodies may have advantages over heteroconjugates if they maintain the half-life of conventional antibodies. However, the process of producing panels of bispecific antibodies to cover a wide range of tumor biased cell-surface antigens is labor intensive. Nor does it necessarily take advantage of other available killing systems. For maximum benefit ECR should allow lysis of tumor cells without harm to normal bystander cells or the effector cells (11) while preserving other anti-tumor effector mechanisms.

To obviate these disadvantages we have constructed a bispecific antibody by fusion of two hybrid myelomas—one secreting antibody specific for CD3 and the other specific for immunoglobulins containing the Igk-1b rat light chain allotype. *In vitro* this bispecific antibody is capable of causing lysis of a range of antibody-coated target cells, and, as for direct ECR, such killing can be induced by low concentrations of antibodies.

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MATERIALS AND METHODS

Monoclonal Antibodies. Two bispecific antibodies, SHN20.12 and LHC49.18, were constructed for use in direct and indirect ECR, respectively. SHN20.12 (11) was produced from parental hybridomas secreting antibodies specific for human CD3 (clone YTH12.5, rat IgG2b, λ light chain) and for mouse Thy-1 [clone YBM29.2, rat IgG2c (12)]. LHC49.18 was produced by cell fusion from parental hybridomas secreting antibodies specific for CD3 (clone YTH12.5 as above) and for the rat Igk-1b immunoglobulin light chain allotype [clone RG11/15.5, a mouse IgG2a antibody (13)]. We have adopted the suggested nomenclature for the rat immunoglobulin κ light chain allotypes as given by Gutman *et al.* (14). Target cell-specific monoclonal antibodies all bearing κ light chains of the Igk-1b allotype were as follows: YBM29.2, described above; YTS154.7, a rat IgG2b specific for mouse Thy-1 (12); YBM42.2, a rat IgG2a specific for murine leukocyte common antigen, the mouse equivalent of human CD45 (15); YTH54.12, a rat IgG2b monoclonal antibody specific for human CD45 (16). Several antiglobulin-specific monoclonal antibodies were used in the detection of the bispecific and target cell-specific monoclonal antibodies: YA9/36.39 is a rat IgM monoclonal antibody specific for mouse IgG2a and IgG2b (17); NORIG7.16 is a mouse IgG2a monoclonal antibody specific for rat IgG2b heavy chain (18). In addition, the CD16 (FcRlo)-specific monoclonal antibody CLB/FcRgranI (19), a mouse IgG2a, was used as described to inhibit killer cell (K-cell) killing mediated by rat IgG2b monoclonal antibodies (11).

Fusion Protocol. The procedure allows any two antibody-producing hybridomas to be fused and has been described elsewhere (11). Briefly, 5×10^6 to 3.5×10^7 cells of one parental hybridoma that have been previously rendered hypoxanthine/aminopterin/thymidine (HAT) sensitive by selection for a hypoxanthine phosphoribosyltransferase-negative variant were fused at a 1:1 or 10:1 ratio, using 1 ml of a 50% (wt/vol) solution of polyethylene glycol, with the second parental hybridoma cells that had been pretreated with a lethal dose of 10 mM iodoacetamide. Excess polyethylene glycol was washed out and the cells were plated at concentrations from 8×10^5 per ml to 2×10^5 per ml into 24-well plates in bicarbonate-buffered Iscove's modified Dulbecco's medium (IMDM) supplemented with 5% (vol/vol) fetal calf serum. After 24 hr in culture, hybrid hybridomas were selected for in medium containing HAT.

Screening for Bispecific Antibody-Producing Hybrid Hybridomas. The detection of SHN20.12 has been described (11). A variety of assays were used to isolate LHC49.18. CD3 specificity was detected with immunofluorescent staining of the human T-cell line HPB-ALL. Functional anti-Igk-1b

Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; CTL, cytotoxic T lymphocyte; ECR, effector cell retargeting; K cell, killer cell.

activity was demonstrated by an enzyme-linked immunoadsorbent assay (ELISA) using rat IgG2c, Igk-1b antibody (YBM29.2) adsorbed to microtiter wells. The monoclonal antibody specific for rat Igk-1b was detected with biotinylated anti-mouse IgG2 (YA9/36.39). The immunoadsorbent assay was modified to demonstrate the presence of the CD3-specific (YTH12.5) IgG2b heavy chain in association with the rat Igk-1b-specific (RG11/15.5) heavy and light chains by using biotinylated anti-rat IgG2b (NORIG7.16) instead of anti-mouse IgG2 (YA9/36.39). Cells from wells containing hybrid antibody were cloned twice on semisolid agar, and LHC49.18 was selected for further studies.

Enrichment of the Bispecific LHC49.18. Bispecific antibody LHC49.18 was enriched from a 50% saturated ammonium sulfate cut of hybridoma culture supernatant containing 2% fetal calf serum in bicarbonate-buffered IMDM. Precipitated proteins were redissolved in water and dialyzed into 50 mM malonate buffer at pH 4.8 containing 0.5% (wt/vol) betaine. The sample was loaded onto a TSKSP-5PW 7.5 × 75 mm Ultropac HPLC ion-exchange column (LKB, Bromma, Sweden), and proteins were eluted with a 0–1 M NaCl gradient. The collected fractions were subjected to the various assays described above to determine which fractions contained parental type CD3 or Igk-1b monospecific antibody and which contained the bispecific LHC49.18, as summarized in Table 1.

Cell Lines. The murine thymoma line BW-5147 and 3-day Con A-stimulated blasts from BALB/c mouse spleen were used as target cells. The human CD3-positive T-cell leukemia line HPB-ALL was used in immunofluorescence.

Effector Cells. Mononuclear cells were isolated on Ficoll/Hypaque gradients from defibrinated human peripheral blood. Cells were resuspended to 1×10^6 per ml in IMDM with 5% (vol/vol) fetal calf serum and activated in tissue culture flasks that were pretreated with a mitogenic CD3 antibody (YTH12.5) at 50 μg/ml in phosphate-buffered saline [137 mM NaCl/2.68 mM KCl/8.10 mM Na_2HPO_4/1.47 mM KH_2PO_4 (PBS)] for 24 hr and washed four times with PBS to remove unbound monoclonal antibody. Cells were cultured for 3 days at 37°C and transferred to untreated flasks for 3–7 days of expansion in IMDM with 5% (vol/vol) fetal calf serum and 10 units of recombinant interleukin 2 per ml (Cetus). Activated cells were tested for ADCC- and CD3-triggered cell-mediated lysis before use in the retargeting experiments. The procedure consistently gave greater cytotoxic T lymphocyte (CTL) and K-cell activation when compared to effectors stimulated with optimal concentrations of the mitogen phytohemagglutinin (20).

Cytotoxicity Measurement. To measure direct ECR, mouse cell lines expressing Thy-1 were labeled with 300 μCi (1 Ci = 37 GBq) of sodium [^{51}Cr]chromate in 150 μl of bicarbonate-buffered IMDM for 1 hr at 37°C. To measure indirect ECR, various targets were labeled as above with an additional 1-hr incubation with 500 μl of appropriate culture supernatants containing anti-target cell antibody in excess. Target cells were washed and 10^4 viable cells were added in a volume of 50 μl per well to U-bottom 96-well plates (Costar, Cambridge, MA). Activated effector cells were washed to remove interleukin 2 and, where appropriate, were treated with a 1:600 dilution of CLB/FcRgranI (CD16) containing ascites fluid for 30 min at room temperature to block ADCC. They were then added to target cells in a volume of 50 μl per well at either a constant or variable effector-to-target ratio. Antibodies from hybridoma culture supernatants at concentrations of between 10 and 100 μg/ml were added in a final volume of 100 μl per well. The plates were incubated at 37°C for 4 hr and 100 μl of supernatant per well was collected for determination of radioactivity released. Cell lysis was estimated by the specific ^{51}Cr release and was calculated as $[(a - b)/(c - b)] \times 100$, where a = experimental release, b = spontaneous release, and c = maximum release from target cells.

RESULTS

Feasibility of Indirect ECR. The potency of the indirect method is demonstrated in Fig. 1, where the target is the Thy-1-bearing mouse thymoma cell line BW-5147. LHC49.18 (CD3/Igk-1b specific) hybridoma supernatant could indeed mediate ECR against antibody-coated but not uncoated targets. In contrast, SHN20.12, a bispecific antibody made between CD3 and the same Thy-1-specific antibody (YBM29.2, rat IgG2c Igk-1b) used to coat the target cells, lysed antibody-coated and uncoated targets. However, as expected, lysis of antibody-treated targets was less because of the bound anti-Thy-1 monoclonal antibody that blocked the binding of the bispecific antibody. No lysis of antibody-coated or uncoated targets was seen with the parental antibodies or artificial mixtures of the relevant antibodies.

The effect of varying the effector-to-target ratio using constant concentrations of the two bispecific antibodies is shown in Fig. 2. Indirect and direct ECR were able to induce potent lysis of target cells at effector-to-target ratios as low as 2:1, with maximum lysis being achieved at ratios above 16:1. Again, no lysis was seen in the presence of an artificial mixture of the parental antibodies.

To demonstrate the versatility of indirect ECR, a variety of target cells and of cell-surface-specific monoclonal antibod-

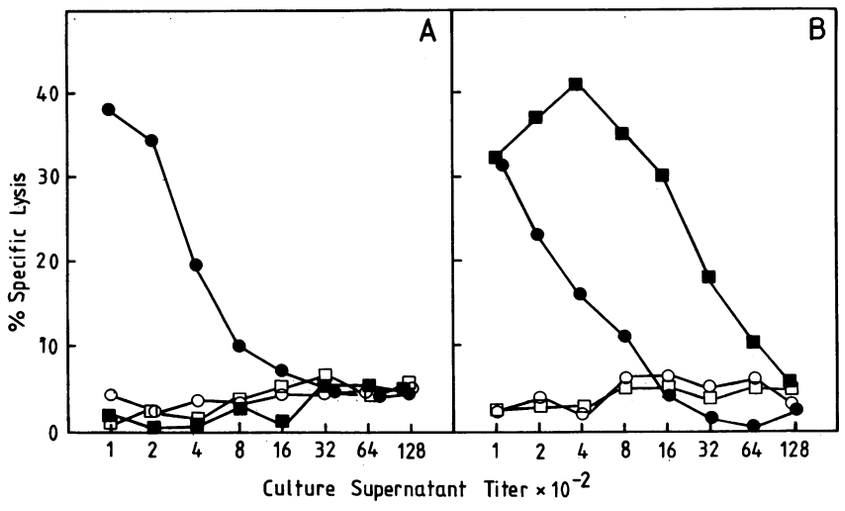

FIG. 1. Titration of antibody concentration in indirect and direct ECR. Radiolabeled Thy-1-expressing mouse thymoma targets were preincubated with medium only (*A*) or medium containing a rat IgG2c anti-Thy-1 antibody, YBM29.2 (*B*). These target cells were then set up in microtiter wells containing dilutions of bispecific antibody or control antibodies together with activated effectors at an effector-to-target ratio of 10:1. After 4 hr at 37°C, 100 μl of supernatant was harvested to determine specific release of radioactivity and hence estimate cell lysis. Antibodies were bispecific LHC49.18 (■), a 1:1 mixture of LHC parental antibodies (□), bispecific SHN20.12 (●), and a 1:1 mixture of SHN parental antibodies (○).

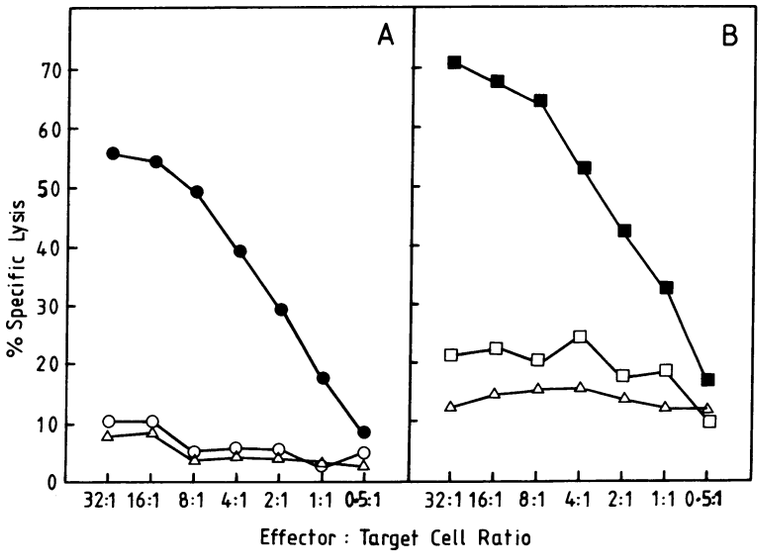

FIG. 2. Comparison by effector cell titration. Uncoated target cells (*A*) or rat IgG2c-coated target cells (*B*) were mixed with activated effector cells, at different cell ratios, in the presence of constant concentrations of bispecific antibody or control antibodies. After a 4-hr incubation, the specific cell lysis was determined. Antibodies were bispecific LHC49.18 (■), a 1:1 mixture of LHC parental antibodies (□), bispecific SHN20.12 (●), and a 1:1 mixture of SHN parental antibodies (○) and medium control (△).

ies of different isotypes were examined. The use of mouse target cells allowed us to avoid situations in which effectors and targets shared antigens; additionally, it facilitated comparison between direct and indirect ECR. In Fig. 3 *A–C* (and also Figs. 1, 2, and 4) it can be seen that culture supernatant of LHC49.18 can direct CTLs to kill various mouse target cells coated with rat Igk-1b monoclonal antibodies. No lysis of targets was observed in the absence of the bispecific antibody nor with an artificial mixture of the two parental antibodies.

ECR Is Independent of ADCC. We have previously reported that anti-target antibodies of the rat IgG2b isotype were able to mediate ADCC by human K cells (4). ADCC could be prevented by blocking the Fc receptor on the effector K cells using the CD16 monoclonal antibody as described (11) (Fig. 4). In the presence of a rat IgG2b anti-target monoclonal antibody and with untreated effectors, ADCC could be demonstrated as lysis in the absence of bispecific second antibody (Fig. 4*A*). The amount of lysis was not increased upon addition of bispecific antibody LHC49.18 (Fig. 4*A*). In the presence of CD16 antibody, ADCC was blocked completely (Fig. 4*B*). However the blocking CD16 antibody did not prevent lysis by indirect ECR observed when the bispecific antibody LHC49.18 was added (Fig. 4*B*). When the anti-target antibody was of an isotype that did not elicit ADCC, the indirect ECR was unaffected by the presence or absence of the CD16 antibody (Fig. 4 *C* and *D*). One interesting point to note from Fig. 4*A* is that ADCC was also blocked by the mixture of LHC parental antibodies. This was due to cold target blocking by unlabeled effectors coated with the rat IgG2b CD3 parental antibody as described (11).

Indirect ECR Against Human Targets. Antibody present in the culture supernatant of LHC49.18 was enriched by fractionation using HPLC ion-exchange chromtography. Table 1 displays the properties of the various fractions obtained. It can be seen that the bulk of the indirect ECR activity coincides with the detection of mixed molecules in fractions 5 and 6. Fraction 1 contains no immunoglobulin; fraction 2 is largely composed of RG11/15.5, and fraction 3 contains only rat IgG2b with CD3 specificity. The light chain composition on sodium dodecyl sulfate/PAGE was also consistent with these observations (data not shown). Fraction 5 was then tested at 125 ng/ml for its ability to elicit indirect ECR on human peripheral blood mononuclear cells by effector cells generated from the same individual (Fig. 5). A CD16 monoclonal antibody was included (as above) to prevent any ADCC. The antibody-coated target cells were clearly killed in a specific manner in the presence of purified bispecific antibody. No lysis of uncoated targets or of antibody-coated targets in the absence of bispecific antibody was observed.

DISCUSSION

By the use of a cell-fusion protocol, which allows two different hybrid myeloma cell lines to fuse to form a hybrid hybridoma, we have produced a bispecific antibody with dual specificity for CD3 and for rat immunoglobulin κ light chains. The CD3-producing rat–rat hybridoma secreted a rat IgG2b antibody with a λ light chain, whereas the other parental

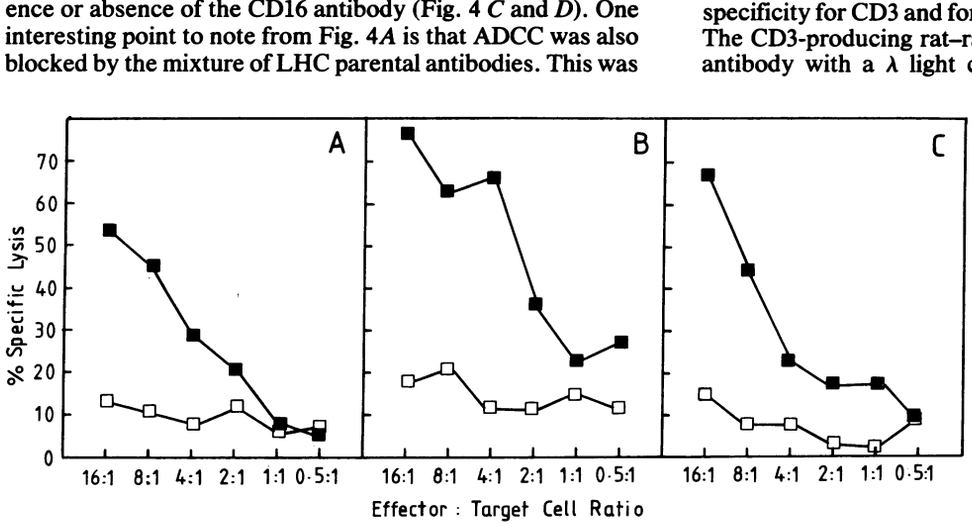

FIG. 3. Lysis of different antibody/target combinations. In addition to the antibody/target cell combination shown in Figs. 1 and 2, the mouse thymoma target cells were incubated with a rat IgG2a antibody to the leukocyte common antigen (*A*), and mouse spleen cell-derived Con A-stimulated blasts were also incubated with the rat IgG2c anti-Thy-1 antibody (*B*) or the rat IgG2a antibody to the leukocyte common antigen (*C*). Experimental conditions were as for Fig. 2. Antibodies used were bispecific LHC49.18 (■) and a 1:1 mixture of LHC parental antibodies (□).

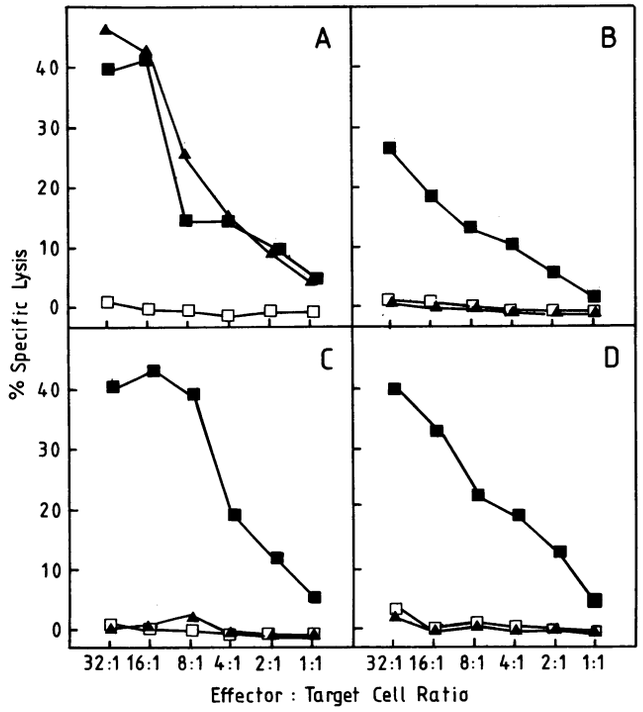

FIG. 4. Comparison of indirect ECR and ADCC. Mouse thymoma target cells were incubated with either a rat IgG2b anti-Thy-1 antibody (*A* and *B*) or a rat IgG2c anti-Thy-1 antibody (*C* and *D*). Antibodies were then tested for indirect ECR in the presence (*B* and *D*) or absence (*A* and *C*) of a CD16 monoclonal antibody that blocks ADCC. Antibodies used were LHC49.18 (■) and a 1:1 mixture of LHC parental antibodies (□) and medium control (▲).

hybridoma cell was of mouse–mouse origin and secreted a mouse IgG2a antibody. Therefore this particular functional bispecific antibody demonstrates that rat and mouse heavy chains can associated with each other. This represents a useful addition to the list of previously described bispecific antibodies of either entirely rat (11, 21–23) or mouse (9, 10, 24–27) origin. This bispecific antibody has the property of being able to cross-link CD3 to immunoglobulins bearing the rat Igk-1b allotype and has the potential to mediate indirect ECR.

ECR provides a way by which cytotoxic T-cell killing can be exploited in serotherapy without a requirement for antigen recognition. Though ECR is major histocompatibility system unrestricted, it is still antigen specific in nature in the sense that CTL will lyse only cells possessing serological target antigen so that "innocent bystander" cells are not killed (10, 28, 29). Indirect ECR looks to be an extremely versatile adaptation of ECR requiring the production of relatively few bispecific antibodies that can then be used in conjunction with panels of tumor-targeting monoclonal antibodies.

Indirect ECR has potential applications in serotherapy. An individual undergoing tumor therapy is often immunocompromised as a result of the disease and also of the treatment. It would be advantageous to direct the potential of the patient's CTL and helper T-cell populations using bispecific antibodies. A possible treatment would entail removing some peripheral blood lymphocytes, activating the T cells briefly with monoclonal antibody as we have described, and expanding the effectors in interleukin 2 and other relevant lymphokines in a manner analogous to lymphokine-activated K-cell production (30). Appropriate monoclonal antibodies or combinations of monoclonal antibodies could be used to target the tumor cells for lysis by these activated effectors, which could be reintroduced into the body several hours later in the presence of the bispecific antibody, after excess anti-tumor monoclonal antibody has been cleared.

It has previously been shown that cells can be killed when Fc-receptor bearing cells are cross-linked to CTLs by the Fc portion of the CD3 antibody (31). To avoid this complication, either Fc receptors must be blocked or the Fc portion of the bispecific antibody must be selected or treated to render it inactive (11). Unfortunately, such procedures preclude exploitation of ADCC. Indirect ECR can bypass the problem if

Table 1. Analysis of fractions of LHC49.18 antibody

Fraction*	Elution (M NaCl)	ELISA† Detection of r2b/r2b‡	ELISA† Detection of anti-k-1b m2a§	ELISA† Detection of anti-k-1b r2b¶	Binding to human T cells‖	ECR activity** (titer)
1	0.05	0.03	0	0	−	<50
2	0.16	0.07	0.57	0.04	−	<50
3	0.2	0.57	0.09	0.11	±	<50
4	0.22	0.63	0.07	0.44	+ + +	1,250
5	0.26	0.66	0.09	1.29	+ + +	31,250
6	0.27	0.77	0.13	1.18	+ + +	31,250
PBS		0	0.08	0	−	—
LHC49.18††		0.7	0.32	0.95	NT	1,250

*1:50 dilution of fraction in PBS/1% heat-inactivated rabbit serum/1% bovine serum albumin/0.1% azide.

†Values are means of duplicates representing the reaction product of conversion of *o*-phenylenediamine by the enzyme horseradish peroxidase (measured by adsorption at 492 nm).

‡Positive results indicate the presence of parental CD3 monoclonal antibody with rat γ2b heavy chains (r2b/r2b).

§Positive results indicate the presence of RG11/15.5 heavy (m2a) and light chains (by detection of antigen binding to a plate coated with rat k-1b-bearing immunoglobulin).

¶Positive results indicate the presence of mixed molecules of rat γ2b heavy chain (r2b) together with RG11/15.5 (m2a) heavy and light chains (by detection of antigen binding to a plate coated with rat k-1b-bearing immunoglobulin).

‖Staining of HPB-ALL cells by immunofluorescence [detection with biotin-anti-rat immunoglobulin (1:100) and streptavidin-fluorescein isothiocyanate (1:100)]. NT, not tested.

**Values give the reciprocal dilution of the LHC49.18 fraction that gave half-maximal lysis of YBM29.2-coated BW3147 cells in the presence of activated T cells (effector-to-target ratio = 10:1).

††1:50 dilution of hybridoma supernatant in PBS.

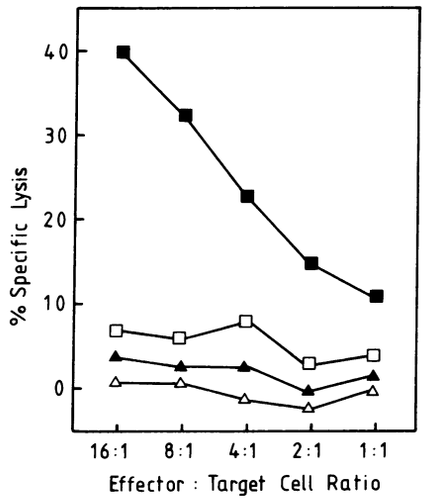

FIG. 5. Indirect ECR using human targets and autologous effectors. Human peripheral blood mononuclear cells were isolated from a healthy donor and were activated *in vitro*. These cells were then used as effectors at different effector-to-target cell ratios with radiolabeled fresh peripheral blood mononuclear cells derived from the same donor. Cell lysis was determined for antibody-coated (rat IgG2b CD45, YTH54.12) and uncoated target cells in the presence or absence of fraction 5 of LHC49.18 (see Table 1) at a concentration of 125 ng/ml. Conditions were uncoated targets with no second antibody (△), uncoated targets followed by bispecific LHC49.18 (□), CD45-coated targets with no second antibody (▲), and CD45-coated targets followed by bispecific LHC49.18 (■).

a bispecific antibody is chosen that cannot bind Fc receptors, whereas the accompanying anti-target monoclonal antibody can be of an isotype that does and can permit ADCC (e.g., rat IgG2b).

For human therapy, it will be important to achieve complete purification of the bispecific antibody away from the bivalent CD3 monoclonal antibody. Bivalent CD3 antibody has been shown to induce reciprocal killing between CD3-positive effector cells *in vitro* (10) and has also been used for immunosuppression *in vivo* (32). Additionally, any bivalent CD3 monoclonal antibody may induce capping and modulation of CD3 antigen from the surface of activated effectors, thus rendering them ineffective for ECR. The bispecific antibody is functionally monovalent for CD3 and should not modulate the antigen, particularly if the Fc region does not bind to Fc receptors (20).

Though many reports of ECR have concentrated on CD3/Ti-specific hybrid antibodies and heteroaggregates, it is apparent that effector cells other than CTL can be "retargeted." In an early demonstration of ECR a heteroaggregate possessing specificity for CD16 and 2,4-dinitrophenol was able to induce CD16-bearing effectors to kill a variety of 2,4-dinitrophenol-modified erythrocytes and tumor targets (29). In another example, a heteroaggregate with specificity for FcRI and chicken erythrocytes was described that increased monocyte killing of chicken erythrocytes (33). It should be equally possible to adapt indirect ECR to these other effector cell systems.

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