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Bispecific antibodies that mediate killing of cells infected with human immunodeficiency virus of any strain

(CD4/antibody to CD3/cytotoxic T cells/human immunodeficiency virus-infected HeLa cells)

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ABSTRACT **Although AIDS patients lose human immunodeficiency virus (HIV)-specific cytotoxic T cells, their remaining CD8-positive T lymphocytes maintain cytotoxic function. To exploit this fact we have constructed bispecific antibodies that direct cytotoxic T lymphocytes of any specificity to cells that express gp120 of HIV. These bispecific antibodies comprise one heavy/light chain pair from an antibody to CD3, linked to a heavy chain whose variable region has been replaced with sequences from CD4 plus a second light chain. CD3 is part of the antigen receptor on T cells and is responsible for signal transduction. In the presence of these bispecific antibodies, T cells of irrelevant specificity effectively lyse HIV-infected cells *in vitro*.**

The immune system normally deals with a viral infection in two ways: cytotoxic T cells kill infected cells, and antibodies neutralize free virus particles, preventing them from infecting other cells. Although cytotoxic T cells (1, 2) and neutralizing antibodies (3, 4) are present in human immunodeficiency virus (HIV)-infected individuals, they do not halt the lethal course of AIDS. This may be due to the high antigenic variability of HIV (5, 6). It has also been argued that an antibody to the viral envelope protein gp120 would neutralize virus of any strain, if it blocked the epitope that makes contact with the CD4 molecule, the receptor for HIV on the cell surface. Soluble CD4 itself is a surrogate for such an antibody (7–11) and, when attached to the constant region of immunoglobulin (12, 13), exhibits effector functions of immunoglobulin, such as complement binding (13), Fc receptor binding (12, 13), antibody-dependent cellular cytotoxicity, and placental transfer (14). But antibodies are generally rather ineffective in killing cells and thus fail to stop the production of new virus particles.

The CD8-positive T lymphocytes of AIDS patients maintain cytotoxic function (15), and there is ample evidence that cytotoxic T cells of irrelevant specificity can be focused onto and stimulated to kill target cells by means of bispecific antibodies (reviewed in ref. 16). Because they express integral viral proteins on the surface, cells infected by retroviruses lend themselves particularly well to the bispecific antibody approach. Indeed, it has previously been shown that human peripheral blood lymphocytes can kill HIV-infected cells *in vitro* in the presence of monoclonal anti-gp120 antibodies chemically cross-linked to anti-CD3 monoclonal antibodies (17). In an attempt to provide an improved immunotherapy for AIDS, we have combined the property of CD4 to bind to the gp120 of any HIV strain with the T-cell activation property of antibodies to CD3 to activate T cells of any specificity.

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MATERIALS AND METHODS

Plasmid Construction. The plasmid integrated in the genome of the transfectoma was generated by cutting out an *Eco*RI–*Sfi* I fragment from the CD4·γ1 plasmid (12), filling the ends, and blunt end-ligating the fragment into the *Xba* I site of the pcDEB vector (18), which contains the hygromycin-resistance gene.

Biosynthetic Labeling and Electrophoresis of Immunoglobulin Chains. Cells were cultured at a density of 4×10^6 per ml for 2 hr in methionine-free RPMI 1640 medium containing 10% dialyzed fetal calf serum and 3.7 MBq of [^{35}S]methionine per ml. The cells were washed in phosphate-buffered saline (PBS) and lysed with NET buffer (0.05 M Tris·HCl, pH 7.4/150 mM NaCl/5 mM EDTA/0.1% NaN_3/0.5% Triton X-100). Immunoglobulin was precipitated either from cell lysates or from culture supernatants using rabbit antiserum to mouse IgG (Cappel Laboratories) followed by *Staphylococcus aureus*; the precipitate was boiled in sample buffer [62.5 mM Tris·HCl, pH 6.8/10% glycerol/0.002% bromphenol blue/5% 2-mercaptoethanol/2.5% sodium dodecyl sulfate (SDS)] and analyzed by SDS/polyacrylamide gel electrophoresis (PAGE). The gels were treated with EN^{3}HANCE solution (New England Nuclear) and fluorographs were taken from dried gels. Samples run under nonreducing conditions were boiled in sample buffer without mercaptoethanol.

Anti-CD4 Affinity Chromatography. Culture supernatant of the transfectomas was centrifuged 10 min at $800 \times g$, filtered, and concentrated 3-fold on a hemodialysis membrane (Fresenius, Bad Homburg, F.R.G.). Anti-CD4 resin (K.S.S., unpublished) was incubated with the concentrate at 4°C for 16 hr and packed to form a column. The column was washed with 100 ml of buffer (0.2 M NaCl/0.01 M phosphate, pH 7.4). Bispecific antibodies or CD4·γ1,κ tetramers were eluted with 100 mM glycine at pH 2.5 and collected in 1 M Tris·HCl buffer at pH 8. The eluted immunoglobulins were dialyzed against PBS and analyzed by SDS/PAGE under reducing and nonreducing conditions. The gels were stained with Coomassie blue.

Cells. MX66 cells were obtained from H. Spits (DNAX, Palo Alto, CA). They were propagated in Yssel's medium (19); it consists of Iscove's modified Dulbecco's medium, of which 1 liter was supplemented with 3.024 g of $NaHCO_3$, 2.5 g of bovine serum albumin (Sigma), 1.8 μg of 2-aminoethanol (Merck), 40 mg of transferrin (Boehringer Mannheim), 5 mg of insulin (Sigma), 2 mg of linoleic acid (Sigma), 2 mg of oleic acid (Sigma), and 2 mg of palmitic acid (Sigma). Human type AB serum was used instead of fetal calf serum. Every 7 days, the MX66 cells were stimulated in the following way: 2×10^5 MX66 cells, 10^6 irradiated peripheral blood mononuclear

Abbreviations: HIV, human immunodeficiency virus; H, heavy; L, light.

cells, and 10^5 irradiated JY cells were suspended in 1 ml of medium containing 50 ng of phytohemagglutinin (Burroughs Wellcome), and the cell mixture was cultured in 24 cluster plates (Linbro/Flow Laboratories) at 37°C and 5% CO_2. After 2 days, 20 units of interleukin 2 (IL-2) (Boehringer Mannheim) was added. After 4 days, cells were washed once in RPMI 1640 medium and placed back into medium supplemented with IL-2. Killing assays were performed 5–7 days after stimulation.

$HeLaT4^+$, a HeLa cell line stably transfected with human CD4 (20), was provided by D. Littman (University of California, San Francisco). The cells were grown in Dulbecco's modified Eagle's medium (DME medium) supplemented with 10% fetal calf serum, sodium pyruvate (1 mM), 2-mercaptoethanol (0.05 mM), L-glutamine (4 mM) and penicillin/streptomycin (100 units/ml). HUT 78 cells were obtained from the American Type Culture Collection, and were propagated in RPMI 1640 medium supplemented as for DME medium.

M-T301 cells were obtained from E. Rieber (University of Munich, F.R.G.) (28); they were grown in RPMI 1640 medium.

Viral Infection. HIV-BRU (originally LAV-1) (21) was obtained from F. Barre-Sinoussi (Pasteur Institute, Paris). Viral stocks were prepared from acutely infected HUT 78 cells as described (22), and clarified culture supernatant was frozen in aliquots at −80°C. $HeLaT4^+$ cells (2×10^6) were resuspended in 2 ml of freshly thawed viral stock and incubated on ice for 1 hr. Five milliliters of medium was added and cells were placed in an incubator at 37°C at 7% CO_2. On day 5 of infection, cells were subcloned by limiting dilution (0.3 per well).

Cytoplasmic Immunofluorescence. For cytoplasmic immunofluorescence, 10^5 cells were centrifuged on a slide, fixed in 95% ethanol, air dried, and rehydrated in PBS supplemented with 1% bovine serum albumin and 0.1% sodium azide (PBSF). The slides were incubated 10 min with 15 μl (5 μg/ml) of mouse monoclonal antibody to HIV-1 core protein p25 (22), washed three times in PBSF, and incubated with 15 μl of fluorescein isothiocyanate-labeled goat antibody to mouse IgG2b, diluted 1:20 (Fisher). The cells were then analyzed by fluorescence microscopy.

Membrane Immunofluorescence. Cells (5×10^5) were resuspended in 15 μl (5 μg/ml) of CD4·γ1,κ antibody (CD4·γ1) or in 15 μl (20 μg/ml) of irrelevant mouse IgG1 antibody (MOPC 21) and incubated 20 min on ice. Cells were washed three times in PBSF and resuspended in 15 μl of fluorescein isothiocyanate-labeled goat antibody to mouse IgG1, diluted 1:20 (Fisher). After 20 min cells were washed twice in PBSF and then washed in PBS and fixed in 0.4% formaldehyde. The cells were analyzed either by fluorescence microscopy or by flow cytometry.

Chromium Release Assay. To 2×10^6 target cells, suspended in 200 μl of RPMI 1640 medium containing 20% fetal calf serum and 0.5 mM Hepes, was added 300 μCi of ^{51}Cr (sodium ^{51}chromate, New England Nuclear; 1 Ci = 37 GBq), and the cells were labeled for 90 min at 37°C and 7% CO_2. The cells were centrifuged, and pellets were resuspended and split in aliquots of 15 μl of antibody solution. After 15 min of incubation at 37°C and 7% CO_2, the cells were washed three times, adjusted to 2×10^4 cells per ml in RPMI 1640 medium containing 10% fetal calf serum and 0.5 mM Hepes, and dispensed into U-shaped 96-well microtiter plates (100 μl per well). One-hundred microliters of an effector cell suspension was added, and the plates were centrifuged 5 min at $200 \times g$ and incubated for 4 hr at 37°C and 7% CO_2. Then the plates were centrifuged 10 min at $600 \times g$, 100 μl of supernatant was transferred to counting vials, and the radioactivity was measured in a γ counter. The percentage of lysed cells (resulting in ^{51}Cr release) was calculated using the formula: % lysis = [(cpm experimental release − cpm spontaneous release)/(cpm maximal release − cpm spontaneous release)] × 100. The maximum release was determined after lysis of the target cells by adding 100 μl of 1 M HCl. The spontaneous release was determined by incubating the target cells with 100 μl of medium only. Assays were performed in triplicates.

RESULTS AND DISCUSSION

A Transfectoma Secreting Bispecific Antibodies. We cloned the gene encoding the fusion polypeptide CD4·γ1, described by Capon *et al.* (12), into a vector that allows expression in mouse cells and transfected it into hybridoma M-T301, which secretes a γ1,κ anti-human CD3 antibody. The transfectoma obtained in this way expresses molecules of specificities to gp120 and CD3. It secretes, among other products, the desired bispecific antibodies (Fig. 1). The anti-gp120 activity is conferred by the CD4·γ1 chain; it consists of the first four immunoglobulin-like domains of CD4 fused to the whole constant region of mouse γ1 chain. HIV cannot mutate out of the binding capability of CD4 without losing its ability to infect cells by means of their membrane CD4 molecule. The anti-CD3 activity is conferred by a heavy (H) × light (L) chain pair of the M-T301 antibody. When added in polymerized form this antibody can activate T cells (23). Fig. 2*A* shows an autoradiograph of biosynthetically labeled intracellular proteins precipitated with antibody to mouse IgG (lanes 2 and 3). A 92,000 molecular weight polypeptide representing CD4·γ1 is precipitated from the transfected (lane 3), but not from the untransfected, cell line (lane 2). Some of the CD4·γ1 is secreted (Fig. 2*B*) in association with the κ chain of M-T301, this dimer being linked to the H × L chain pair of M-T301. In addition, there are other combinations of the three polypeptide chains present in the transfectoma; this can be deduced from the molecular weights of the unreduced molecules (Fig. 2*C*, lane 4). Furthermore, we have rerun lane 4 of Fig. 2*C* under reducing conditions and confirmed that some of the molecules contain CD4·γ1, H, and L chains (Fig. 2*D*).

Purification of Bispecific Antibodies. From the supernatant of the transfectoma, we then purified bispecific antibodies by

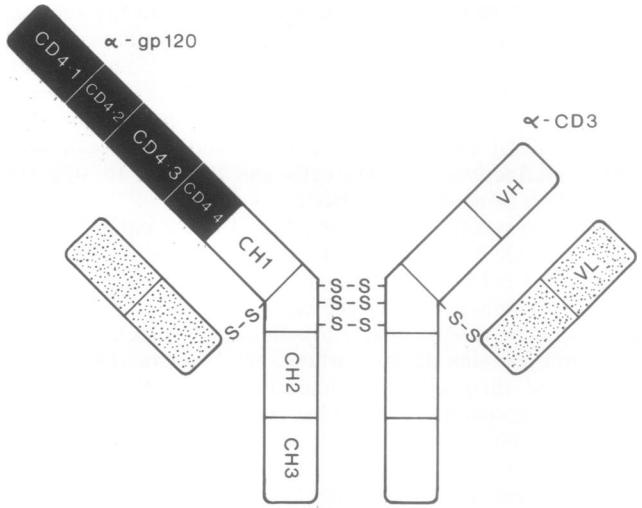

FIG. 1. Schematic representation of our bispecific antibody. The left arm of the molecule consists of the fusion protein CD4·γ1 linked to κ light (L) chain by a disulfide bridge; the right arm consists of a γ1,κ pair. The left arm binds to HIV gp120 of any strain. The right arm of the bispecific antibody binds to CD3, a component of the T-cell receptor and, thereby, activates the cytotoxic T cell. CD4·1, CD4·2, CD4·3, and CD4·4, immunoglobulin-like domains of CD4; CH1, CH2, and CH3, constant region domains of γ1 chain; VH, variable region of γ1 heavy (H) chain; VL, variable region of κ chain.

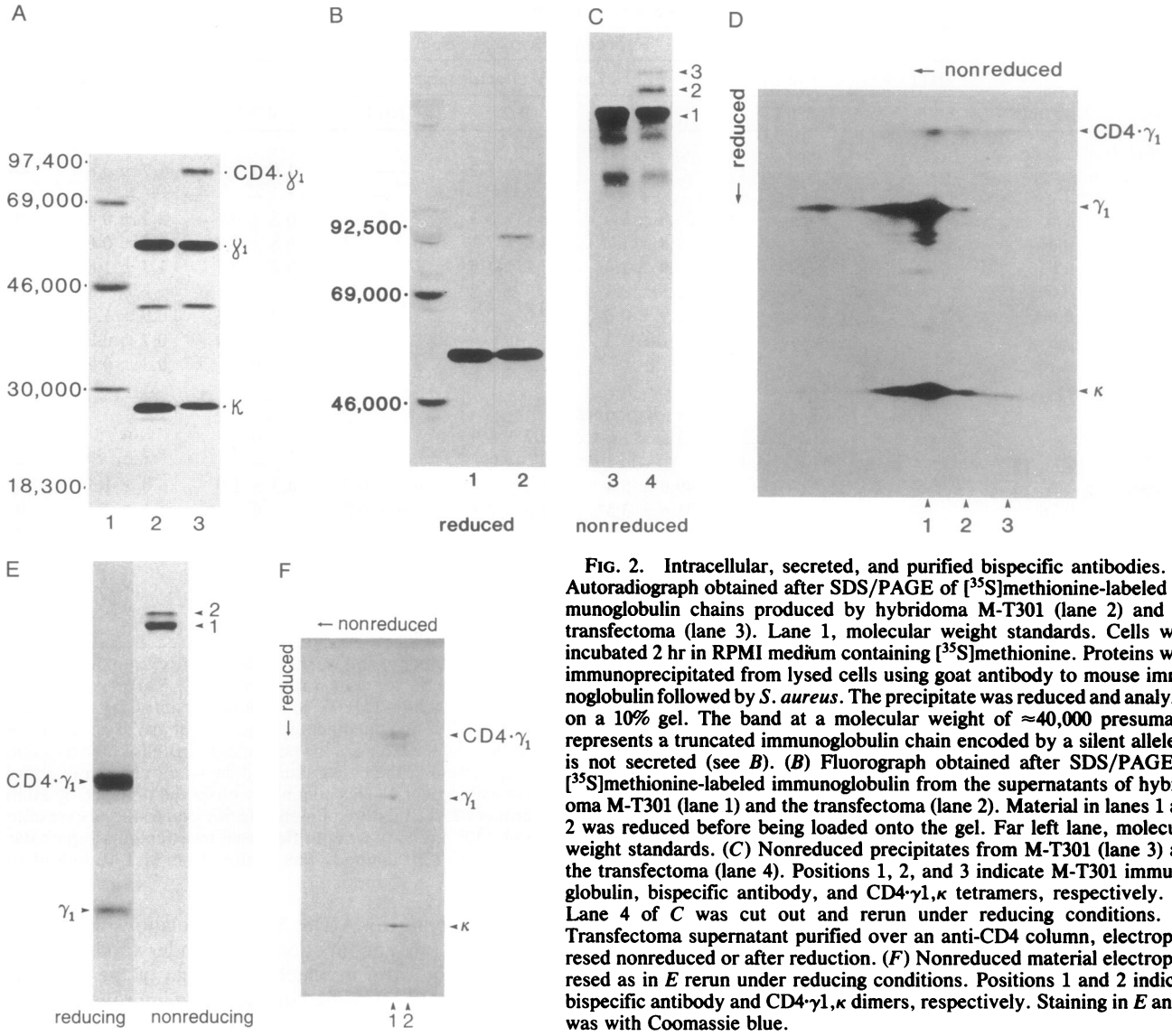

FIG. 2. Intracellular, secreted, and purified bispecific antibodies. (*A*) Autoradiograph obtained after SDS/PAGE of [^{35}S]methionine-labeled immunoglobulin chains produced by hybridoma M-T301 (lane 2) and the transfectoma (lane 3). Lane 1, molecular weight standards. Cells were incubated 2 hr in RPMI medium containing [^{35}S]methionine. Proteins were immunoprecipitated from lysed cells using goat antibody to mouse immunoglobulin followed by *S. aureus*. The precipitate was reduced and analyzed on a 10% gel. The band at a molecular weight of ≈40,000 presumably represents a truncated immunoglobulin chain encoded by a silent allele; it is not secreted (see *B*). (*B*) Fluorograph obtained after SDS/PAGE of [^{35}S]methionine-labeled immunoglobulin from the supernatants of hybridoma M-T301 (lane 1) and the transfectoma (lane 2). Material in lanes 1 and 2 was reduced before being loaded onto the gel. Far left lane, molecular weight standards. (*C*) Nonreduced precipitates from M-T301 (lane 3) and the transfectoma (lane 4). Positions 1, 2, and 3 indicate M-T301 immunoglobulin, bispecific antibody, and CD4·γ1,κ tetramers, respectively. (*D*) Lane 4 of *C* was cut out and rerun under reducing conditions. (*E*) Transfectoma supernatant purified over an anti-CD4 column, electrophoresed nonreduced or after reduction. (*F*) Nonreduced material electrophoresed as in *E* rerun under reducing conditions. Positions 1 and 2 indicate bispecific antibody and CD4·γ1,κ dimers, respectively. Staining in *E* and *F* was with Coomassie blue.

binding them to and eluting them from an affinity column with a monoclonal anti-CD4 antibody (K.S.S., unpublished) to remove the bivalent anti-CD3 antibodies. On electrophorograms of this material on a polyacrylamide gel roughly equal proportions of two kinds of molecules can be seen: a bivalent monospecific tetramer consisting of CD4·γ1 and κ and the desired bispecific antibody with the composition κ, CD4·γ1, γ1, κ (Fig. 2*E*). Thus, the CD4·γ1 chain is linked to κ and pairs with the H × L chain half of the IgG1 molecule of M-T301 (Fig. 2*F*). Fortunately, the rather large CD4·γ1 is not sterically hindered from being linked to the γ1 chain, probably because of the flexibility of the hinge region, which allows the amino ends of the polypeptides to move apart. Since the κ chain is also not hindered from forming a disulfide bridge with the CD4·γ1 chain, the desired molecule is secreted. Generally, immunoglobulin H chains not linked to L chain are held back by immunoglobulin H chain binding protein (BiP; ref. 24) in the endoplasmic reticulum (25). Because hybridomas usually produce only 1.5 times more L chain than H chain (26), the amount of L chain in our transfectoma is not optimal to release all H chain from BiP. Transfecting another copy of the L chain of M-T301 into the transfectoma ought to increase the secretion of complete molecules.

Chromium Release Assay. To test the ability of the bispecific antibodies to mediate killing of HIV-infected cells by nonspecific cytotoxic T cells we developed a chromium release assay. As target cells, we used CD4-expressing HeLa cells (designated HeLaT4$^+$) (20) that we had infected with HIV. The percentage of infected cells was assessed by immunofluorescence with a monoclonal antibody to viral p24 (22, 27) for testing expression in the cytoplasm and the monospecific CD4·γ1,κ antibody for testing expression of membrane gp120. From the bulk culture we subcloned lines in which >90% of the cells stably expressed these proteins (J. Berg, K.S.S., and M.W., unpublished work).

As effector cells, we used clones of the human cytotoxic T-cell line MX66, which is specific for influenza virus matrix protein when presented on human cells expressing the histocompatibility antigen A2. At an effector:target ratio of 1:1 the MX66 cells did not significantly kill the CD3-positive HUT 78 cell line; however, when M-T301 antibody, which is specific to CD3, was added, good killing was seen (Table 1). This confirms that antibody M-T301 is able to mediate the killing of noncognate target cells, by activating MX66 cells and linking them to these targets. Thus, this would seem to be a suitable system for testing the bispecific antibodies.

Bispecific Antibodies Mediate Killing of Cells Infected with HIV. Table 1 shows that our bispecific antibodies are strikingly effective in promoting the killing of HIV-infected cells *in vitro*. In experiment 1, HeLaT4$^+$ cells, whether HIV-infected or not, were not killed by effector cells alone. However, when we added our bispecific antibodies at a

Table 1. Efficacy of bispecific antibody in killing HIV-infected target cells *in vitro*

Antibody	Conc., μg/ml	% specific ^{51}Cr release from target cells							
		HUT 78 effector:target		HeLa-LAV effector:target			HeLa effector:target		
		0.5:1	1:1	0.5:1	1:1	No E	0.5:1	1:1	No E
				Experiment 1					
None	—	0	0.4 ± 0.2	0	0.7 ± 1.2	—	1.6 ± 1.7	1.7 ± 0.8	—
M-T301	≈2	34.2 ± 2.3	48.3 ± 1.9	—	—	—	—	—	—
Bispecific	1.6	—	—	26.8 ± 3.0	40.3 ± 3.3	0	0.3 ± 0.6	2.2 ± 0.4	0
	0.16	—	—	9.8 ± 1.7	14.6 ± 2.1	0.9 ± 1.2	0.8 ± 0.8	0.5 ± 0.6	0
	0.016	—	—	0.9 ± 1.6	3.2 ± 1.5	0	0.4 ± 0.1	1.7 ± 1.8	0
	0.0016	—	—	2.3 ± 0.9	0.9 ± 1.2	0	0	0	0
	0.00016	—	—	0.9 ± 0.6	2.0 ± 2.2	0	0.2 ± 0.3	0.9 ± 1.4	0
Mixture	1.6	—	—	0	0	0	0.2 ± 0.9	0.7 ± 0.8	0
	0.16	—	—	0	0.2 ± 1.8	0	0	0.3 ± 0.9	0
				Experiment 2					
None	—	0.3 ± 0.8	1.5 ± 0.5	3.5 ± 1.6	8.2 ± 1.9	—	0	0	—
M-T301	≈2	53.3 ± 1.2	64.6 ± 4.0	—	—	—	—	—	—
Bispecific	1.6	—	—	49.0 ± 5.4	67.2 ± 3.2	0.8 ± 0.7	4.3 ± 1.9	6.0 ± 1.5	0
	0.16	—	—	31.6 ± 3.5	44.6 ± 2.8	1.0 ± 0.8	0	1.1 ± 2.1	0
	0.016	—	—	10.7 ± 0.4	16.0 ± 2.5	0.4 ± 1.3	0	0.8 ± 2.0	0
	0.0016	—	—	3.1 ± 2.0	5.7 ± 0.2	0	0	2.7 ± 1.3	0
	0.00016	—	—	1.9 ± 1.7	7.0 ± 1.0	0	0	0	0
Mixture	1.6	—	—	11.1 ± 0.5	17.5 ± 1.2	0	0	0	0
	0.16	—	—	6.6 ± 0.5	11.3 ± 1.1	0.1 ± 0.7	0	0	0

Target cells were incubated with antibodies at one or more concentrations (or with no antibody) and washed; cells of the cytotoxic T-cell line MX66 (effector cells) were added at a ratio of 0.5:1 or 1:1 or as a control, medium with no effector cells was added (no E). Targets: HeLa cells expressing CD4 and infected with HIV (HeLa-LAV); not infected (HeLa); or allogeneic cell line HUT 78. Antibodies: anti-CD3 monoclonal antibody M-T301; bispecific antibodies with one arm specific for CD3 (derived from M-T301) and the other arm specific for gp120 (derived from CD4·γ1); a 1:1 mixture of M-T301 and CD4·γ1 (antibody mixture). The values in the body of the table are the percentages of cells killed (resulting in ^{51}Cr release). Spontaneous release was always <15% of maximal release. In experiment 2, the background killing—i.e., chromium release in the presence of effector cells but in the absence of antibodies—is higher than in experiment 1. This commonly observed nonspecific killing by T-cell clones is thought to depend on culture conditions. Furthermore, in experiment 2, the mixture of monospecific antibodies also resulted in some killing of the infected, but not the uninfected, cells. In experiment 2, the M-T301 antibody was purified from transfectoma supernatant and presumably contained small amounts of bispecific antibodies. In experiment 1, the M-T301 antibody was purified from M-T301 hybridoma supernatant and thus must have been free of such contamination.

concentration of 1.6 μg/ml, 26.8% of infected cells were killed at the low effector:target ratio of 0.5:1, whereas the uninfected cells were not killed. At an effector:target ratio of 1:1, 40% of the infected, but only 2% of uninfected cells, were killed. A mixture of equal parts of bivalent CD4·γ1,κ tetramers and M-T301 antibodies did not cause killing (below 0.7%). The effect of the bispecific antibodies was diminished at a lower concentration and was absent at 16 ng/ml. However, in experiment 2, this concentration was still effective (in another experiment not shown, 1.6 ng/ml showed some effect). As mentioned above, the bispecific antibody preparation contained an equal amount of monospecific bivalent CD4·γ1,κ tetramers, which do not promote the killing but, which, due to their superior avidity (two binding sites vs. one), might actually hinder the binding of bispecific antibodies to the target cells. Further purification of the bispecific antibodies should yield preparations that are somewhat more efficient. To alleviate the necessity for tedious purification, we might select mutations in the γ chains that would allow only heterologous pairing—i.e., V_H·γ1 with CD4·γ1.

CONCLUSION

We have shown here that the specificity of the CD4·γ1 fusion polypeptide for gp120 can be combined with the cytotoxicity of killer T cells, the agents meant to destroy infected cells. The decisive advantage of this combination is that it will work against any HIV strain still able to invade cells by combining with CD4. Thus, its therapeutic potential is not limited by the rapid variation characteristic of HIV.

Although the experiments described here were all performed *in vitro*, we are obviously motivated by the problem of finding a therapy for AIDS. Since the relationship between antibody structure and function is well understood, there are several obvious ways in which the design of the bispecific antibodies might be modified to attempt to improve function. However, the pathogenesis of AIDS is not fully understood, and it is not certain that eliminating gp120-expressing cells will affect the progression of the disease. It is not understood why the immune system of AIDS patients, which is depleted of CD4-positive cells, is rendered ineffective even though only 1 of 1000 CD4-positive T cells actually expresses HIV (29). Having demonstrated the effectiveness of bispecific antibodies *in vitro*, only clinical testing can determine whether they might be of therapeutic value.

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