Correction. In the article "Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses" by David J. Lane, Bernadette Pace, Gary J. Olsen, David A. Stahl, Mitchell L. Sogin, and Norman R. Pace, which appeared in number 20, October 1985, of Proc. Natl. Acad. Sci. USA (82, 6955-6959), the authors request that the following correction be noted. On p. 6957, the first sentence of the paragraph beginning on line 9 of the left-hand column should read "Reverse transcriptase has been used previously to copy rRNA templates (14-17); Bachellerie and his colleagues (17) have used a restriction fragment primer applicable to the 28S rRNA of multiple eukaryotic species."

Correction. In the article "Hybrid hybridoma producing a bispecific monoclonal antibody that can focus effector T-cell activity" by Uwe D. Staerz and Michael J. Bevan, which appeared in number 5, March 1986, of Proc. Natl. Acad. Sci. USA (83, 1453-1457), the authors request that the following addition be noted. This work was performed while Uwe D. Staerz was a graduate student in the Department of Biology, University of California, San Diego, La Jolla, CA 92093, and it forms a part of his Ph.D. thesis.
Hybrid hybridoma producing a bispecific monoclonal antibody that can focus effector T-cell activity
(hybrid antibody/cytotoxic T lymphocyte/T-cell receptor)

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ABSTRACT

Previous studies have shown that heteroconjugates of monoclonal antibodies in which one of the component antibodies is directed at the T-cell receptor and the other is directed against any chosen site can focus effector T cells to function at the targeted site. We report here the production of a hybrid hybridoma cell line, H1.10.1.6, which secretes large amounts of a bispecific hybrid antibody of the IgG2a class, that can focus T-cell activity. The parental hybridoma lines for the secondary fusion were F23.1, which secretes an antibody specific for an allotypic determinant on the T-cell receptor of most mouse strains, and 19E12, secreting an anti-Thy-1.1 antibody. The bispecific hybrid antibody was partially purified by hydroxylapatite chromatography and characterized by isoelectric focusing. It efficiently targets Thy-1.1-expressing tumor cells for lysis by F23.1 receptor-positive cytotoxic T-cell clones in vitro. Such hybrid antibodies produced by hybrid hybridoma cell lines may have application in the therapeutic targeting of tumors or sites of viral infections for attack by T cells.

The receptor on T cells that confers the specificity of their response to foreign antigen in association with a self-histocompatibility antigen has recently been identified. Two chains, each of 40–50 kDa, called α and β, form a disulfide-bonded heterodimer. Both chains possess variable (V) and constant (C) regions that are encoded by separate V region, diversity (D) region, joining (J) region, and C region genes (1–5). This heterodimer is noncovalently associated on the cell surface with three invariant polypeptides referred to as the T3 complex (6–8).

Many different types of monoclonal antibodies (mAbs) have been raised against the T-cell receptor complex. Some of these react only with the particular T-cell clone used as immunogen—i.e., antidiotopic (or antiidiotype) mAbs (9–11). A number of murine mAbs generated against human T-cell lines recognize 1–6% of human T cells (12, 13). mAbs directed against an allotypic determinant expressed by one family of murine Vβ regions react with about 20% of T cells in many inbred strains (14, 15). Antibodies directed against the T3 complex react with all mature T cells (6–8). All antibodies directed against the T-cell receptor–T3 complex can inhibit the function of T cells and, when insolubilized—on a bead, for example—can stimulate T-cell proliferation (10, 11, 16, 17).

It has recently been shown that these same antireceptor mAbs can serve as target structures to focus T-cell activity at a certain site. The B-cell hybridomas producing mAbs against a cytotoxic T lymphocyte (CTL) receptor are sensitive targets for lysis by the CTL (18, 19). Furthermore, we and others have shown that antidiotopic and antiidiotype mAbs covalently coupled to any cell surface will render that cell sensitive to lysis by a CTL expressing a receptor recognized by the mAb (20, 21). Thus, one can bypass the need for antigen or histocompatibility antigen recognition with an antibody-combining site. Finally, heteroconjugates of mAbs, one of which recognizes the T-cell receptor, can focus a T cell to act at a site targeted by the other binding site of the bispecific heteroconjugate (20, 22).

We have proposed that bispecific mAbs may be used in vivo for the therapeutic targeting of sites for T-cell attack. One of the antibody sites should be anti-T-cell receptor and the other should be directed against a viral determinant expressed on infected cells or against a tumor-associated antigen. Chemical heteroconjugates of mAbs may be quickly removed from the circulation in vivo and may not readily access certain target sites. We report here that recombinant F(ab')2 dimers of mAbs produced by reduction/reoxidation and a true 7S hybrid antibody produced by a hybrid hybridoma cell line can efficiently target sites for attack by T cells.

MATERIALS AND METHODS

CTL Clones. OE4 and OE25 were isolated by limiting dilution from C57BL/6 (H-2b) spleen cells stimulated with irradiated DBA/2 (H-2d) spleen cells. They were kindly provided to us by Osami Kanagawa (Lilly Research Laboratories, La Jolla, CA). Both were maintained by weekly subculture in RPMI 1640 medium (Microbiological Associates) containing 5% fetal bovine serum, 0.05 mM 2-mercaptoethanol, 5% supernatant of Lewis rat spleen cells stimulated with Con A for 24 hr, and 50 mM α-methyl mannoside. Routinely, 2 × 10⁶ CTL are cultured with 5 × 10⁶ DBA/2 spleen cells irradiated with 2000 rad (1 rad = 0.01 gray) in 2 ml of medium in Costar (Cambridge, MA) 3424 wells. Both CTL clones recognize and lyse H-2d targets, and both express the Thy-1.2 and Lyt-2.2 alloantigens. In addition, the β chain of the antigen receptor of OE4 is recognized by the mAb F23.1. The CTL clone OE25 is not reactive with this mAb.

Target Cells. S. AKR is a T-cell lymphoma derived from an AKR/J mouse that expresses H-2k and the Thy-1.1 alloantigen. EL4 is a T-cell lymphoma derived from a C57BL/6 mouse that expresses H-2b and Thy-1.2. Both lymphomas are maintained in culture in RPMI 1640 medium containing 10% fetal bovine serum.

mAbs. The F23.1 hybridoma was produced by fusion of immunized C57L/J spleen cells with the P3 myeloma (15). The C57L/J spleen donor had been immunized four times with nylon wool-purified T cells of BALB.B mice. The F23.1 mAb is an IgG2a that recognizes the T cell receptor on ≈20% of T cells of most mouse strains, including BALB and C57BL/6. 19E12 is an IgG2a mAb that recognizes the Thy-1.1 alloantigen (23, 24). Ascites fluid was produced in pristane-primed, irradiated (BALB/c × C57BL/6)F₁ mice.

Abbreviations: CTL, cytotoxic T lymphocyte(s); mAb, monoclonal antibody.
Production of the Hybrid Hybridoma. One of the subclones of F23.1 was found to be unable to grow in the hybridoma-selective medium containing hypoxanthine, aminopterin, and thymidine (HAT medium). To select for this phenotype this line was carried for 2 weeks in medium containing 8-azaguanine. It was then subjected to increasing concentrations of ouabain, from 0.01 to 0.1 mM. Thus, we isolated a line of F23.1 that was HAT-sensitive and ouabain-resistant that continued to produce the mAb. This line was fused using polyethylene glycol to the 19E12 hybridoma (HAT-resistant, ouabain-sensitive) in a 1:1 ratio. One day following fusion, the cells were plated out in Costar 96-well microtiter plates in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, HAT, and 0.1 mM ouabain. Two weeks after plating, medium from growth-positive wells was tested for the presence of antibodies. KSH.4.38 is a rat/mouse hybridoma line expressing the F23.1 determinant that was used to test for the presence of this antibody (25). S.AKR lymphoma cells were used to test for the presence of the anti-Thy-1.1 antibody 19E12. Aliquots of supernatant were allowed to react with the cells at 37°C for 30 min; cells were then washed and resuspended in a 1:50 dilution of fluorescein-conjugated goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA). After washing, the cells were fixed in 1% formaldehyde in phosphate-buffered saline and examined on a fluorescence-activated cell sorter (FACS IV, Becton Dickinson). Ninety percent of the supernatants from growth-positive wells that we screened contained both antibody activities. One of the double-positive wells, H1.10, was subcloned and screened in the same manner to yield a stable, double-producer hybrid hybridoma, H1.10.1.1. Ascites fluid was produced in pristane-primed, irradiated (BALB/c × BALB.B)F1 mice.

Purification and Characterization of the Hybrid Antibody. Ascites fluid containing H1.10.1.6 immunoglobulins was dialyzed against phosphate-buffered saline (pH 7.4) and passed through a protein A-Sepharose column (Pharmacia, Uppsala, Sweden). Bound immunoglobulin was eluted with pH 4 citrate buffer (26). Separation of the “parental” immunoglobulins, F23.1 and 19E12, from the hybrid antibody was achieved by chromatography on a hydroxyapatite HPLC column (Bio-Gel HPT, Bio-Rad). Protein A-purified material was loaded on the column in 10 mM sodium phosphate (pH 6.8) and eluted with a gradient of 10–350 mM sodium phosphate buffer (pH 6.8). Chemical characterization of the hybrid and parental immunoglobulins was performed by isoelectric focusing using Pharmalyte 5-8 (Pharmacia). The reduced antibodies were analyzed under denaturing conditions (27). Protein bands were visualized with Coomassie blue (unreduced samples) or with silver staining (reduced samples).

Preparation of Recombinant F(ab')2. Protein A-Sepharose-purified mAbs F23.1 and 19E12 were digested with pepsin at pH 4.1 for 1 hr according to Parham (28). F(ab')2 fragments were purified by chromatography on protein A-Sepharose followed by a Sephacryl S-200 column and their purity was assayed by acrylamide gel electrophoresis. F(ab')2 fragments of both antibodies were reduced with 10 mM mercaptoethanol at pH 6.8 and the column-purified Fab' monomers were recombined at pH 8 under a stream of oxygen (29).

CTL Assay. One hundred microliters of serial dilutions of antibody preparations was placed in the wells of round-bottomed microtiter plates (Costar 3799). One hundred microliters of medium containing 1 × 10⁴ Na51CrO4-labeled target cells (S.AKR or EL4) and 3 × 10⁴ CTL clone (OE4 or OE25) was added to each well. Plates were incubated at 37°C for 4 hr and centrifuged, and 100 μl of the supernatant was removed and assayed for radioactivity. Spontaneous release was measured by culturing labeled target cells in the absence of CTL. Total lysis was determined by using 1% Triton X-100. Percent specific release was calculated as 100 × (experimental release − spontaneous release)/(detergent release − spontaneous release).

RESULTS

Heteroconjugates of mAbs (one of which reacts with the T-cell receptor complex), formed with the bifunctional cross-linking reagent N-succinimidyl-3-(1-pyridyldithio)propionate, have been shown to focus T-cell activity at a target site identified by the other antibody (20, 22). Chemical characterization of the heteroconjugates suggested that dimers and trimers of 7S antibodies could function in this way. To test whether a bivalent, bispecific antibody could focus T-cell activity, we produced recombinant F(ab')2 fragments and a hybrid hybridoma cell line that secretes a hybrid 7S antibody.

The anti-T-cell receptor mAb used in these studies was F23.1, a murine IgG2a that reacts with an allotypic determinant on ~20% of T cells (15). The targeting mAb used was 19E12, a murine IgG2a that reacts with the Thy-1.1 alloantigenic determinant (23, 24). Two CTL clones from C57BL/6 mice specific for H-2d antigens were used, OE4 and OE25. The former expresses a T-cell receptor that reacts with F23.1. The targeted cell line S.AKR expresses Thy-1.1, whereas the control cell line EL4 expresses Thy-1.2.

The data in Fig. 1 suggest that recombinant F(ab')2 fragments, produced by reduction of F23.1 and 19E12 fragments and reoxidation of the mixture, can efficiently target the Thy-1.1-expressing lymphoma S.AKR for lysis by the OE4 CTL clone. Neither fragment alone nor a mixture of them caused any lysis of S.AKR target cells. Further experiments have shown that for lysis to occur, (i) the CTL must express the allotypic determinant recognized by F23.1 and (ii) the target cell must express Thy-1.1 (data not shown).

By fusing the hybridoma cell lines F23.1 and 19E12, we isolated a hybrid hybridoma, H1.10.1.6, which secreted both anti-T-cell receptor and anti-Thy-1.1 antibody. Although the screening of the original hybridoma fusion and subsequent subclonings was done in fluorescence assays, the important question was whether the subclones were secreting any hybrid antibody capable of focusing T-cell activity. Fig. 2

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**Fig. 1.** Recombinant F(ab')2 dimers of F23.1 and 19E12 mAb can target 51Cr-labeled S.AKR cells expressing Thy-1.1 for lysis by F23.1-positive OE4 CTL. F(ab')2 fragments of F23.1 (△), 19E12 (●), a mixture of the two (○), or recombinant (□) were assayed for the ability to cause lysis of S.AKR targets by OE4 CTL.
Fig. 2. Immunoglobulin secreted by the hybrid hybridoma H1.10.1.6 can target S.AKR cells for lysis by OE4 effector cells. Unfractionated tissue culture supernatant of H1.10.1.6 cells (.), protein A-Sepharose-purified immunoglobulin at 2 mg/ml prepared from ascites fluid of mice bearing H1.10.1.6 (v), F23.1 (λ), 19E12 (β), or a 1:1 mixture of F23.1 and 19E12 immunoglobulin (δ) was assayed for the ability to target S.AKR cells for lysis by OE4 CTL.

Fig. 3. The CTL must express the receptor allotypic determinant recognized by F23.1 for lysis to be induced. (A) 31Cr-labeled S.AKR target cells were incubated with the indicated dilutions of immunoglobulin (10⁸ dilution at 2 mg/ml) from H1.10.1.6 (open symbols) or F23.1 (solid symbols) in the presence of OE4 CTL (λ, α) or OE25 CTL (γ, ω). (B) S.AKR targets incubated in the presence of 10 μg of phytohemagglutinin with OE4 CTL (α) or OE25 CTL (γ).

Fig. 4. The target cell must express Thy-1.1 for lysis to occur. OE4 effector CTL were incubated with S.AKR (α) or EL (λ) target cells in the presence of dilutions of H1.10.1.6 immunoglobulin at 2 mg/ml (A) or phytohemagglutinin at 10 μg/ml (B).

matography on hydroxyapatite separated F23.1 and 19E12 quite cleanly. An elution profile of the protein A-Sepharose-purified secretory products of H1.10.1.6 is shown in Fig. 5. Three pools of the major peaks seen in Fig. 5A were made, concentrated, and rerun under the same conditions (Fig. 5B–D). Peak I (Fig. 5B) runs identically to F23.1 mAb, and peak III (Fig. 5D) coincides with the 19E12 mAb. Peak II (Fig. 5C) is thus the putative hybrid antibody. Functional evidence consistent with this suggestion is shown in Fig. 6. Peak I has little activity in inducing lysis of S.AKR targets by OE4 CTL; peak III has some activity but on a weight basis is <1% as active as peak II. In accord with this, it is clear from the hydroxyapatite chromatography that peak III is somewhat contaminated with the material in peak II (Fig. 5D). It is interesting to note that peak II can cause significant lysis of the S.AKR targets by a 3-fold excess of OE4 CTL at a protein concentration as low as 2 mg/ml. Chemical char-

FIG. 5. Fractionation of protein A-Sepharose-purified immunoglobulin produced by the hybrid hybridoma H1.10.1.6. The hydroxyapatite column was loaded in 10 mM sodium phosphate (pH 6.8) and eluted with a gradient of 10–350 mM sodium phosphate (pH 6.8). The three shaded fractions indicated in A were pooled, concentrated, and run under the same conditions: B, peak I; C, peak II; D, peak III. Peak I eluted at 26 min (80 mM), peak II at 37 min (115 mM), and peak III at 44 min (136 mM).

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acterization of the putative hybrid antibody peak from the hydroxylapatite column was performed by isoelectric focusing under nonreducing and reducing conditions (Fig. 7). The starting mAbs, F23.1 and 19E12, are distinct by this technique (Fig. 7, lanes 1 and 2). The HPLC-purified hybrid is quite unlike either parent under nonreducing conditions (Fig. 7A, lane 5) but on reduction can be shown to contain all of the bands present in an artificial mixture of F23.1 and 19E12 (Fig. 7B, compare lane 5 with lane 3). Thus, by functional and chemical criteria it appears that the hybrid hybridoma cell line H1.10.1.6 secretes approximately one-third of its total immunoglobulin output as an F23.1/19E12 hybrid antibody that can efficiently target cells that express Thy-1.1 for lysis by a CTL that expresses the allotypic determinant recognized by the F23.1 mAb.

**DISCUSSION**

In this paper we have described two methods of constructing bispecific antibodies that can target sites for attack by T cells. Both methods may be more useful for *in vivo* applications of T-cell focusing than constructing heteroconjugates of mAbs with chemical crosslinkers (20, 22) because of their longer survival in the circulation and better penetration to extravascular sites.

Recombinant F(ab')2 reagents have been constructed previously for applications in histochemical staining (30). Two different antibody-producing cells have also been fused together previously, as in the original description of the hybridoma technology (31), myeloma–myeloma fusions (32), and recently Milstein and Cuello have fused a hybridoma producing an antiperoxidase mAb with somatostatin immune spleen cells to produce a hybrid antibody useful in histochemistry (33, 34).

We fused together two established hybridomas, one HAT-sensitive, ouabain-resistant, and the other HAT-resistant, ouabain-sensitive. The factors that affect the yield of the desired bispecific, hybrid antibody secreted by the hybrid hybridoma have been discussed (33, 34) and are summarized here. If the rate of production of the two pairs of heavy (H) and light (L) chains is the same, and if the formation of HL pairs shows no homologous or heterologous preference (i.e., L1H1, L2H2, L1H2, and L2H1 form equally), and if H-H pairing is also random, then the yield of the desired bispecific hybrid antibody, L1H1\_H2L2, is 12.5% of the total immunoglobulin. In actual fact, however, there is likely to be a homologous preference in LH pairing (35). If this preference were absolute, and H-H pairing random, then the yield of the desired bispecific hybrid rises to 50% of the total immunoglobulin production. To maximize the chances of random H-H association, we deliberately chose parental antibodies of the same subclass, IgG2a. This probably made the physical separation of the parental and recombinant antibodies more difficult, and, indeed, the two parental immunoglobulins F23.1 and 19E12 were not separable by chromatofocusing or DEAE-Sephadex chromatography. However, the two were well resolved on hydroxylapatite chromatography (Fig. 5).

Since our electrophoretic analysis of the column fractions was not sufficient to estimate the degree of heterologous LH pairing, the middle peak from the chromatographic step, which contains the desired hybrid, may not be pure but may contain other, undesired species, such as L1H\_H2L4, and other forms. We suspect that the actual yield of the desired hybrid is somewhere between 12.5% and 50% of total immunoglobulin production.

It is apparent from the titration of the targeting activity of the chromatographic pools shown in Fig. 6 that the hybrid hybridoma mAb is extremely efficient in this regard. Thus, a protein concentration of 2 ng/ml of pool II is sufficient, in a 4-hr assay, to cause the lysis of 30% of the targeted lymphoma cells, S.AKR, by the CTL clone OE4 at a CTL:target ratio of 3:1. It is also clear that the presence of the parental mAbs (F23.1, which binds the CTL receptor only, and 19E12, which binds only to the Thy-1.1 determinant on the target cell) does not efficiently inhibit the ability of the bispecific hybrid mAb to induce specific lysis of the targets. Thus, whole culture supernatant of H1.10.1.6 and the total, protein A-purified material from ascites fluids function in the targeting assay (Figs. 2–4).

Future work must be directed toward assessing the *in vivo* applications of the hybrid antibody-targeting system. An AKR/J T-cell lymphoma that expresses Thy-1.1 can be titered for growth in AKR/Cum (Thy-1.2) host mice. If the tumor grows in and kills normal AKR/Cum mice, then effector T cells can be induced in the tumor-bearing mice with a strong antigenic challenge, such as a skin graft or a tumor graft of C57BL/6 (H-2b) origin. About 20% of the AKR/Cum anti-H-2b effector T cells express the F23.1 determinant and an attempt can be made to focus these cells on the AKR/J
tumor by injecting the bispecific mAb F23.1/19E12. If the AKR/J tumor does not grow in normal AKR/Cum hosts, then the recipients will have to be immunosuppressed and the induced, effector T cells given adoptively. Obviously, further applications of hybrid antibody targeting lie in focusing T cells to the site of a viral infection.

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