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Immunology

Immunoprecipitation of cell surface structures of cloned cytotoxic T lymphocytes by clone-specific antisera

(cloned T cells/cell surface antigens/T-cell receptor/major histocompatibility complex)

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ABSTRACT **Clones of cytotoxic T lymphocytes (CTL) differ in their specific reactivity with diverse target cell antigens. To learn about the uniqueness of individual CTL clones we injected rats and mice with cloned CTL in an effort to prepare clone-specific antisera and to analyze the CTL surface molecules that were immunoprecipitated by these antisera. Three clones were studied. They were all derived from BALB.B mice and were specific for antigens encoded by the major histocompatibility complex of the *H-2*^{d} haplotype. Antisera raised in rats against individual clones contained antibodies to lymphocyte function-associated antigen type 1 (LFA-1) and inhibited the cytotoxic activity of all of the clones. In contrast, BALB/c and BALB.K mice injected with individual clones consistently yielded alloantisera that were clone specific in their ability to inhibit CTL-mediated lysis of target cells (P815). In addition, these alloantisera immunoprecipitated from extracts of ^{125}I-radiolabeled CTL a disulfide-bonded dimer consisting of ≈45-kilodalton subunits. This dimer resembles the putative T-cell antigen-recognition receptor recently identified in several laboratories. The alloantisera also immunoprecipitated CTL surface molecules that were associated with β_2-microglobulin and that differed in apparent molecular mass (37–38 kilodaltons) in different clones.**

Most immune responses depend in some measure upon the ability of T lymphocytes to discriminate among products of genes of the major histocompatibility complex (MHC) and among a great variety of other antigens (Ag) that are recognized in conjunction with MHC products. The T-cell surface molecules ("receptors") responsible for recognizing these antigens differ in specificity in different T-cell clones but, unlike the Ag-recognition molecules of B lymphocytes, they appear not to be immunoglobulins. Almost nothing more was known about the molecular nature of the T-cell receptors until recently, when several laboratories succeeded in obtaining antibodies (Abs), including monoclonal antibodies (mAbs), that distinguish among T-cell clones (1–4) and immunoprecipitate cell surface molecules that might be T-cell receptors (3–5). We also have been engaged in searching for clone-specific Abs and in doing so have been guided by the experience accumulated in the production of Abs to other well-known structures that are involved in Ag recognition—namely, the variable domains of immunoglobulin molecules. Thus, we used large numbers of cloned T cells to elicit, for comparative purposes, antisera in rats, allogeneic mice, and semisyngeneic mice, producing, respectively, xenoantisera, alloantisera, and what are tantamount to isoantisera. This report focuses primarily on the alloantisera; they have proven particularly informative, because almost every BALB/c AnN and BALB.K mouse immunized with cloned cytotoxic T lymphocytes (CTL) from BALB.B mice has yielded antisera with several interesting properties: (*i*) they inhibit CTL cytotoxic activity and the blocking activity is correlated with the cloned CTL's specificity; (*ii*) one of the non-H-2 CTL surface components precipitated by these sera has turned out to be associated with β_2-microglobulin (β_2m) and to differ in apparent molecular mass in different clones; and (*iii*) another CTL surface molecule precipitated by these antisera resembles the recently described putative T-cell receptor (3–5)—i.e., what appears to be a disulfide-bonded dimer made up of 45-kilodalton (kDa) subunits.

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MATERIALS AND METHODS

Animals. BALB/c AnN (*H-2*^{d}), BALB.K (*H-2*^{k}), BALB.B (*H-2*^{b}), and BALB/c × BALB.B) F_1 (*H-2*$^{d/b}$) mice were bred at the Massachusetts Institute of Technology Center for Cancer Research. Joan Press (Brandeis University) kindly provided B10.A (3R) (K^bD^d) and B10.A (5R) (K^bD^d) mice and Martin Dorf (Harvard Medical School) generously donated C3H.OL (K^dD^k) and C3H.OH (K^dD^k) mice. Inbred rats of the Lewis strain were from Charles River Breeding Laboratories.

Cell Lines. P815 [a DBA/2 (*H-2*^{d}) mastocytoma] and X63.653 (derived from a BALB/c myeloma) were maintained in culture with RPMI 1640 containing 10% fetal calf serum.

Cloned CTL. To produce secondary alloreactive CTL, BALB.B mice were inoculated intraperitoneally (i.p.) with 2 × 10^7 P815 cells (6). One month later their spleen cells were harvested and stimulated in culture with irradiated (2,000 rads; 1 rad = 0.01 gray) BALB/c spleen cells. Three CTL lines (1D, 2C, G4) were cloned by limiting dilution (7) and maintained by weekly stimulation with irradiated BALB/c spleen cells plus supernatants from concanavalin A (Con A) (Vector Laboratories, Burlingame, CA)-stimulated BALB/c or rat spleen cells. Clones 1D and 2C were from the same immunized mouse, and clone G4 was from a different mouse.

Antisera. To prepare the cloned CTL for injection into mice and rats, they were freed of irradiated stimulator cells and debris by centrifugation through Ficoll-Paque and then washed three times with Hanks' balanced salt solution. Rats were injected twice (3 weeks apart) i.p. with 1.5 × 10^7 clone 1D cells. To prepare mouse alloantisera, BALB/c and BALB.K mice were injected i.p. with 5–10 × 10^6 cells (clone 1D) or 10–20 × 10^6 cells (clone 2C) every 2 weeks. Animals were bled from the tail 1 week after each injection. To pre-

Abbreviations: Ab, antibody; Ag, antigen; Con A, concanavalin A; CTL, cytotoxic T lymphocyte(s); E/T, effector-to-target cell ratio; IEF, isoelectric focusing; β_2m, β_2-microglobulin; i.p., intraperitoneally; kDa, kilodalton(s); LFA, lymphocyte function-associated antigen; mAb, monoclonal antibody; MHC, major histocompatibility complex.

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pare what are essentially isoantisera, (BALB/c × BALB.B) F_1 mice were injected i.p. with ≈5 × 10^6 clone G4 cells at 2- to 4-week intervals and bled 1 week after each injection.

Conventional mouse alloantisera to cell surface products of the *H-2*^{b} haplotype (anti-*H-2*^{b}) were prepared by Vivien Igras by biweekly i.p. injections of 2 × 10^7 B10 (*H-2*^{b}) spleen cells into B10.D2 (*H-2*^{d}) mice (B10.D2 anti-B10). A rabbit antiserum to human β_2m, which crossreacted with mouse β_2m, was kindly furnished by Howard Grey (National Jewish Hospital, Denver, CO).

mAbs. Rat–mouse hybridomas were produced by fusion (8) of X63.653 myeloma cells with spleen cells from a rat immunized with clone 1D. Two mAbs, designated 4-16-1 and 5-15-3, were identified as anti-lymphocyte function-associated antigen type 1 (anti-LFA-1). The following mAbs, obtained as hybridoma culture supernatants or ascites fluid, were generous gifts: anti-LFA-1 (M/1.93) (9) was provided by Timothy Springer (Dana–Farber Cancer Institute), anti-Lyt-2 (3.155.2 and 3.168.8) (10) was from Frank Fitch and Marian Sarmeinto (University of Chicago), and anti-*H-2D*^{d} (34-4-215) (11) was from David Sachs (National Cancer Institute).

Cytotoxicity Assays. CTL activity was measured by a standard ^{51}Cr-release assay (12). The ability of antisera or mAbs to inhibit target cell lysis was determined by incubating 50 μl of antiserum (or culture supernatants) at a specified dilution with 1 × 10^4 CTL in 50 μl for 30 min at room temperature. ^{51}Cr-labeled P815 target cells (1 × 10^4 in 100 μl) were then added and incubation was continued 4 hr at 37°C. After cells were pelleted by centrifugation, supernatants were assayed for radioactivity and the percent specific ^{51}Cr release was calculated from 100 × $(a - b)/(t - b)$, where a is ^{51}Cr release in the presence of CTL, b is the spontaneous release from labeled target cells (<10%), and t is the total ^{51}Cr content of the target cells.

Radioiodination of Cells. Cell surface proteins were iodinated with ^{125}I, using lactoperoxidase (13). Briefly, 2–3 × 10^7 cells were washed three times with phosphate-buffered saline and the following were added to the cell pellet: 100 μg of lactoperoxidase, 1 mCi of Na^{125}I (1 Ci = 37 gBq; New England Nuclear), and 50 μl of H_2O_2 (0.03%). The labeled cells were washed three times and extracted with 1 ml of 0.5% Nonidet P-40 in 0.15 M NaCl/0.02% NaN_3/25 μM phenylmethylsulfonyl fluoride/10 mM Tris·HCl, pH 7.2, and, in some instances, 50 mM iodoacetamide.

NaDodSO$_4$/PAGE. Samples were electrophoresed in 10% polyacrylamide gels containing NaDodSO$_4$ (14). In the two-dimensional "diagonal" NaDodSO$_4$/PAGE, nonreduced samples (prepared by boiling bacterial pellets for 5 min) were electrophoresed on cylindrical 7.5% polyacrylamide gels followed by electrophoresis of these gels under reducing conditions (0.7 M 2-mercaptoethanol) in 10% polyacrylamide gels (15). Some samples were analyzed by two-dimensional gel electrophoresis involving isoelectric focusing (IEF) in the first dimension (16, 17). After electrophoresis, gels were fixed, dried, and exposed to Kodak X-Omat XAR film using a Dupont Cronex Lighting Plus intensifying screen.

RESULTS

Specificity of CTL Clones. Because the BALB.B CTL clones were all derived from spleen cells that had been immunized against cells having the *H-2*^{d} haplotype, it was assumed that they recognized *H-2*^{d}-encoded antigens. To define their individual specificities more precisely, they were assayed against a panel of ^{51}Cr-labeled targets, including P815 and Con A blasts prepared from spleen cells of various congeneic mouse strains (see *Materials and Methods*). Each clone lysed only those target cells having the *D* end of the *H-2*^{d} locus; for instance, at a 1:1 effector-to-target cell ratio (E/T) each one caused 30–45% specific ^{51}Cr release from B10.A (3R) Con A blasts (K^bD^d) but no specific release from C3H.OL (K^dD^k) Con A blasts. It was further shown that a mAb to D^d (34-4-215S; ref. 11) blocked cytotoxic activity of clones 1D and G4 almost completely but did not block at all the cytotoxic activity of clone 2C.

Inhibition of Cytotoxic Activity. Rat antisera to clone 1D blocked equally well the cytotoxic activity of this and the other clones (Table 1). In contrast, the inhibitory activity of the alloantisera raised in both BALB/c and BALB.K mice consistently correlated with the clone's specificity: alloantisera to clone 1D blocked the activity of clones 1D and G4 but not the activity of clone 2C, whereas antisera to 2C blocked the activity of this clone but not that of clones 1D and G4 (Table 1, Fig. 1). The correlation between specificity of an alloantiserum's blocking activity and the specificity of the clone used to produce that antiserum was evident in the antiserum from virtually every one of the BALB.K and BALB/c mice after they had received three or four injections of the appropriate BALB.B CTL. Two F_1 mice (BALB/c × BALB.B) (*H-2*$^{d/b}$) required nine injections of clone G4 cells to produce "isoantisera" that blocked CTL activity to only a slight extent; however, this inhibition was also clone specific (i.e., anti-G4 isoantiserum blocked the cytotoxic activity of clone G4 but not that of clone 2C).

The blocking activity of the BALB/c and BALB.K alloantisera was evidently not due to destruction of the BALB.B CTL (shown by trypan blue exclusion and ^{51}Cr-release tests). The failure of P815 cells to significantly adsorb the antiserum's inhibitory activity (data not shown) indicated that inhibition was not due to antibodies that blocked target cells antigens. The inhibitory activity could also not have been due to antibodies to LFA-1 or Lyt-2: not only would these antibodies not be formed in the strain combinations used for the alloimmunizations but, if they had been elicited, they should have blocked all of the clones, regardless of differences in their specificity (Table 1).

Immunoprecipitation of CTL Surface Molecules. As shown in Fig. 2*A*, the BALB/c and BALB.K antisera to clone 1D precipitated from ^{125}I-labeled extracts of clones 1D and 2C

Table 1. Inhibition of target cell lysis by antisera to CTL clones

Cytotoxic T cells		% inhibition by antisera and mAbs						
		Rat antibody			Mouse antibody			
					Antisera to 1D		Antisera to 2C	
Clone	*H-2* specificity	Antisera to 1D	mAb to LFA-1	mAb to Lyt-2	BALB.K	BALB/c	BALB.K	BALB/c
1D	D^d	85.8	80.0	59.3	46.8	24.4	≤0	≤0
G4	D^d	95.9	66.2	30.2	58.0	28.0	≤0	≤0
2C	*D* end of *H-2*^{d}	99.1	89.0	91.4	≤0	≤0	53.8	35.2

Various antisera and mAbs were preincubated with CTL. ^{51}Cr-labeled P815 target cells were then added at an E/T ratio of 1:1. Values shown are % inhibition of specific ^{51}Cr release from target cells by representative antisera at a final dilution of 1:40 (see Fig. 1) or by hybridoma culture supernatants, containing mAbs to LFA-1 or Lyt-2, at a final dilution of 1:4. % inhibition of ≤0 indicates no detectable inhibition—i.e., 0% ± SD (5%) (or slight enhancement, as shown in Fig. 1).

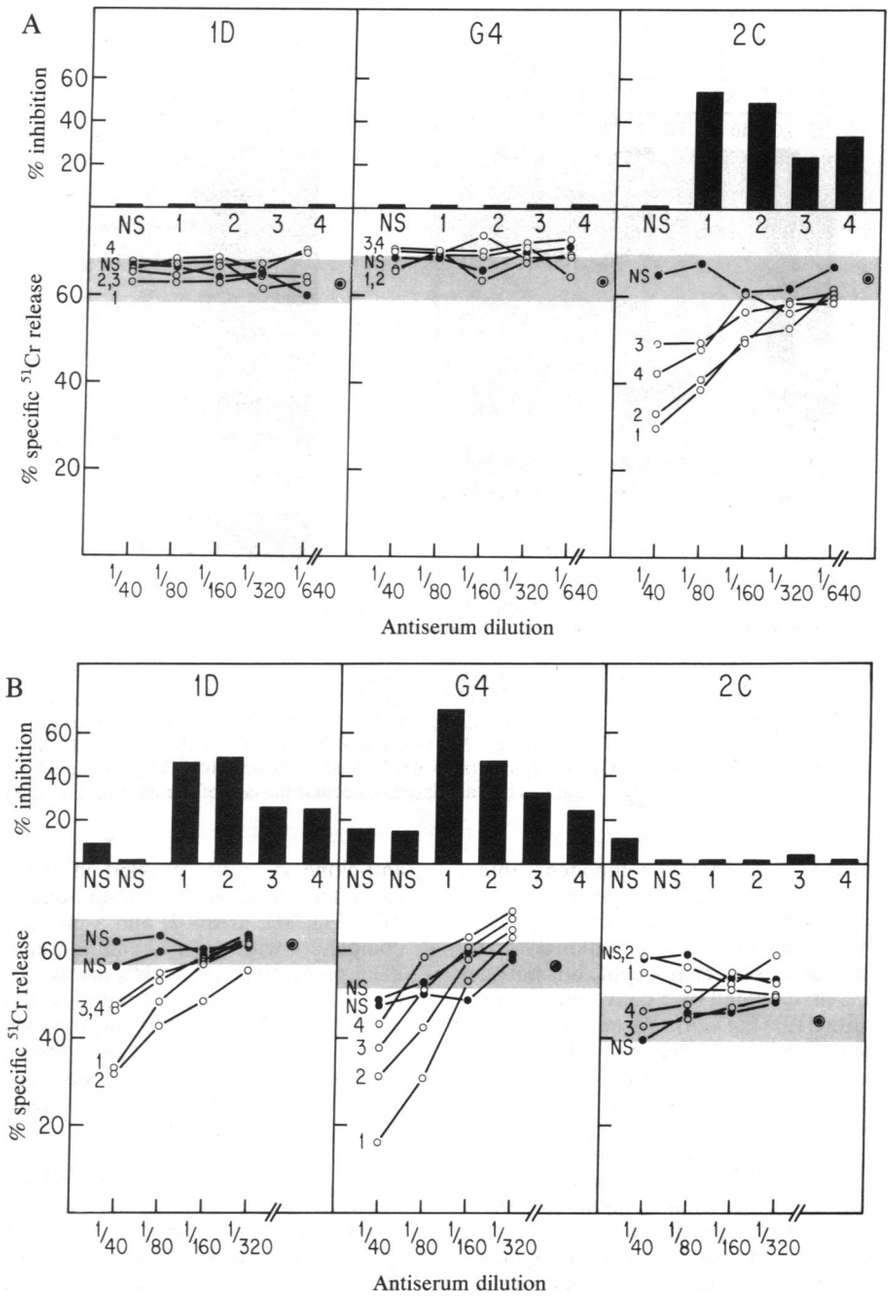

FIG. 1. Inhibition of CTL-mediated lysis of target cells by allogeneic antisera. Fifty microliters of a dilution of normal mouse serum (NS) or various anti-CTL antisera (nos. 1–4) was preincubated for 30 min at about 21°C with 50 μl of 1×10^4 CTL (clone indicated at the top of each panel). After incubation, ^{51}Cr-labeled P815 target cells were added at an E/T ratio of 1:1 and the incubation was continued at 37°C for 4 hr. Values for % specific ^{51}Cr release were computed at each dilution of antiserum or NS and for controls (without any serum, ◉). Shaded areas indicate the standard error of the assays (±5%). The upper half of each panel indicates the % inhibition of a 1:40 dilution of NS or antisera. (*A*) Antisera to clone 2C: from BALB.K (no. 1) or BALB/c (nos. 2–4) mice. (*B*) Antisera to clone 1D; from BALB.K (nos. 1 and 2) or BALB/c (nos. 3 and 4) mice.

four principal components, whose apparent molecular masses were approximately 45, 37–38, 25, and ≤15 kDa. Because the antisera were elicited in H-2 congeneic mice, the 45-kDa band was expected to represent, at least in part, the heavy subunit of conventional class I molecules—e.g., *H-2K*^{b}, *H-2D*^{b}. The 25-kDa band was evidently Con A, as it could be independently precipitated from the cloned CTL extracts with rabbit antibodies to Con A. (Though the CTLs were grown in Con A supernatants containing α-methylmannoside and the cells were washed extensively before being injected into mice, enough lectin evidently remained bound to elicit anti-Con A antibodies.) The low molecular mass band (≤15 kDa) probably contained, or perhaps consisted entirely of, β_2m (which is associated noncovalently with the heavy subunits in class I molecules).

Of particular interest is the 37- to 38-kDa component. In clone 2C its size was 37 kDa, whereas in clone 1D it was 38 kDa. Though this difference is small, it has been consistently observed in many gels and with every one of the alloantisera examined. This difference was also observed with antisera that had been produced against clone 2C in BALB.K and BALB/c mice (Fig. 2*B*). In general, the BALB.K antisera were more effective than the BALB/c antisera in precipitating this component.

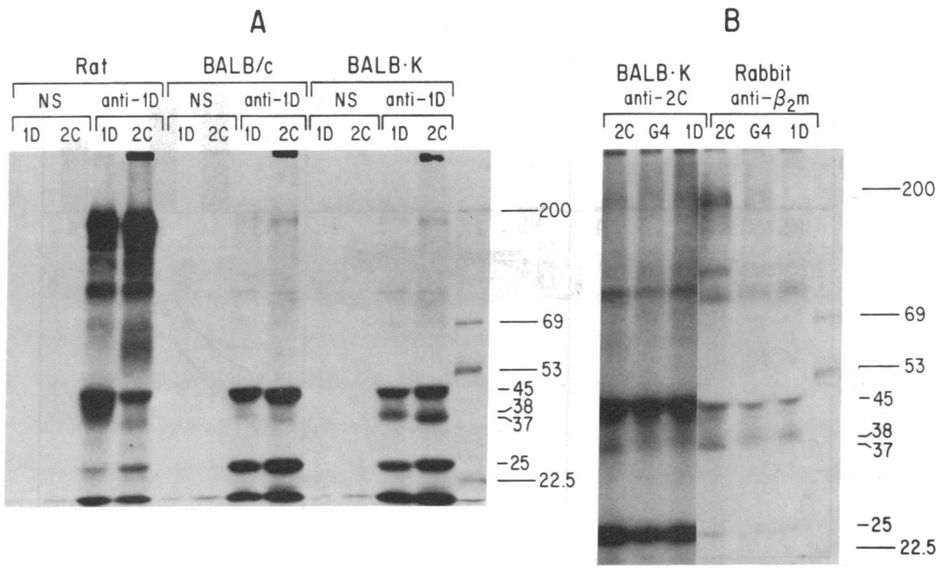

FIG. 2. NaDodSO$_4$/PAGE of CTL surface proteins (^{125}I-labeled) immunoprecipitated with various antisera. CTL clones 1D, 2C, and G4 were ^{125}I-labeled and the Nonidet P-40 cell extracts were analyzed. After the extracts were "precleared" three times with normal rabbit serum and *Staphylococcus aureus* (Cowan strain), they were incubated with 10–20 μl of antiserum or normal serum (NS) for 30 min, followed by incubation with 10 μl of a "developing" antiserum (rabbit anti-rat Ig or rabbit anti-mouse Ig) and finally by incubation with 65 μl of a 20% suspension of the *S. aureus*. After 3 hr on ice, the bacterial pellets were washed three times with extraction buffer and then heated at 100°C in NaDodSO$_4$/PAGE sample buffer containing 2-mercaptoethanol (0.1 M). (*A*) NS and antisera to clone 1D from rats or BALB/c or BALB.K mice were used to immunoprecipitate extracts of clones 1D and 2C. (*B*) BALB.K antisera to clone 2C and rabbit anti-β_2m were used to immunoprecipitate extracts of clones 2C, G4, and 1D. Molecular mass markers are myosin (200 kDa), bovine serum albumin (69 kDa), and immunoglobulin heavy (53 kDa) and light (22.5 kDa) chains. The calculated molecular masses of the immunoprecipitated components are 45, 37–38, and 25 kDa.

To learn more about the 37- to 38-kDa components, precipitates were analyzed by IEF/NaDodSO$_4$/polyacrylamide two-dimensional gel electrophoresis. The patterns of the 45-kDa and 38-kDa material from clone 1D were strikingly similar (Fig. 3*B*). However, the 45-kDa component, but not the 38-kDa component, was precipitated by a conventional anti-*H-2*^{b} serum (B10.D2 anti-B10) (Fig. 3*A*). Based on the similarities in two-dimensional gels, we examined whether the 37- to 38-kDa components, like class I H-2 heavy chains, were associated with β_2m. As shown in Fig. 2*B*, both the 45-kDa and 37- to 38-kDa components from clones 1D, G4, and 2C were precipitated by rabbit antisera to β_2m. Moreover, the mobility of the 37- to 38-kDa component brought down by the anti-β_2m differed in various clones, as noted before with the BALB/c and BALB.K alloantisera.

In contrast to the mouse alloantisera, rat antisera to clone 1D precipitated many additional components, including several of high molecular mass (Fig. 2*A*). The mobilities of the principal ones coincided with those of the α and β chains immunoprecipitated by mAbs to LFA-1 (9), including the two mAbs (4-16-1 and 5-15-3) that we prepared with spleen cells from a rat that had been immunized with clone 1D.

Disulfide-Bonded Surface Structures. Recent reports have suggested that the T-cell receptor is a dimer consisting of disulfide-linked subunits of ≈39–49 kDa each (3–5) and that this dimer may be identified even in total extracts of ^{125}I-labeled cells (5, 15). ^{125}I-labeled extracts of clone 2C were examined accordingly by nonreducing/reducing two-dimensional electrophoresis (Fig. 3*C*). The most prominent disulfide-bonded component consisted of an 80–90 kDa component, which reduced (with 2-mercaptoethanol) to 45 kDa (Fig. 3*C*, arrow 1). A similar component was also identified in total extracts from clones 1D and G4 (not shown). Significantly, a similar component was immunoprecipitated from clone 2C by both the BALB/c and BALB.K antisera to clone 2C (Fig. 3*D*, arrow 1).

Immunoprecipitation of clone 2C extracts with BALB.K anti-clone 2C also revealed in the reducing/nonreducing two-dimensional gel an "on-the-diagonal" component of 55 kDa (Fig. 3*D*, arrow 3) and a prominent "off-the-diagonal" component with reduced and nonreduced sizes of 45 and 55 kDa, respectively (Fig. 3*D*, arrow 2). The former (Fig. 3*D*, arrow 3) was precipitated with late (but not early) bleedings of anti-clone 2C (hence, it was not evident in Fig. 2) and also with some antisera against β_2m; therefore, it appears to be a class I subunit. The off-diagonal component of 55–45 kDa (Fig. 3*D*, arrow 2) was seen repeatedly, though less prominently than in Fig. 3*D*.

DISCUSSION

Antibodies to T-cell surface Ags LFA-1 and Lyt-2 block the cytolytic activity of all CTL clones (in the case of anti-LFA-1) or most of them (in the case of anti-Lyt-2), regardless of the cytolytic cells' specificity for target cells. These Abs probably accounted for most of the blocking activity of the rat antisera raised against the CTL clones in this study. Each alloantiserum, in contrast, had blocking activity that was limited to the clone used to elicit that antiserum or to another clone with the same specificity. Because the clone-specific alloantisera did not destroy the CTL or act by binding to Ags on target cells, the blocking Abs were presumably specific for Ag-binding molecules (receptors) on the CTL.

The apparent receptor specificity of the alloantisera lent particular interest to the radiolabeled molecules immunoprecipitated by these antisera from extracts of ^{125}I-labeled CTL. The antisera were expected to contain Abs to *H-2*^{b}-encoded Ags (of the BALB.B CTL) and to Con A, which is present on CTL as a trace contaminant from the conditioned medium (Con A supernatants) in which the cells were grown. Hence, the precipitation of a 45-kDa component, corresponding to heavy subunits of classical (K^b, D^b) class I molecules, and of a 25-kDa component, corresponding to Con A, was expected. However, the antisera also precipitated a 37-kDa component from one clone (2C) and a 38-kDa component from two

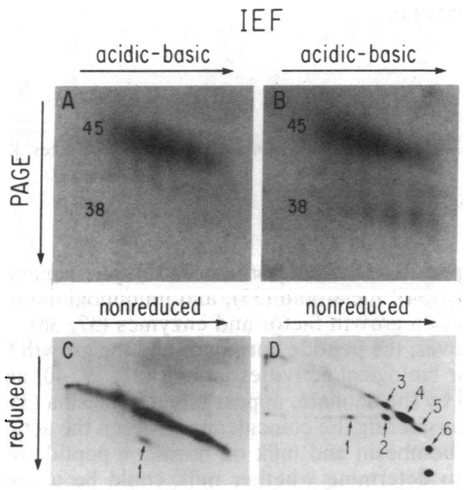

FIG. 3. Two-dimensional gel electrophoresis of ^{125}I-labeled surface structures from cloned CTL. ^{125}I-labeled extracts from clone 1D were immunoprecipitated with B10.D2 anti-B10 antiserum (*A*) and BALB.K anti-1D antiserum (*B*). The precipitates were analyzed in the first dimension by IEF and in the second dimension by NaDodSO$_4$/PAGE on 10% polyacrylamide gels. The positions of the 45-kDa and 38-kDa components (see Fig. 2) are indicated. Total extracts from radiolabeled clone 2C (*C*) and the material precipitated from the extract by BALB.K anti-2C antiserum (*D*) were analyzed by electrophoresis of nonreduced samples in the first dimension (7.5% polyacrylamide gels) followed by reduction (0.7 M 2-mercaptoethanol) and electrophoresis of these gels in the second dimension (10% polyacrylamide gels). Molecular masses of the components indicated by arrows are: ≈84 kDa nonreduced/45 kDa reduced, arrow 1; 55 kDa nonreduced/45 kDa reduced, arrow 2; 55 kDa, arrow 3; 45 kDa, arrow 4; 37 kDa, arrow 5; and 25 kDa, arrow 6. Additional studies using monoclonal anti-Lyt-2 antibodies identified Lyt-2 as a component of ≈35 kDa that also ran off the diagonal (15) but was much less prominent than the component at arrow 1 from clone 2C.

clones (1D and G4). The small difference in size was consistently observed in many gels and, like the cytolytic-blocking activity, it was correlated with a difference in the clones' specificity for Ag on the target cells.

The finding that antisera to β_2m precipitated the 37-kDa component from clone 2C and the 38-kDa component from clones 1D and G4 indicated that these components are class I-like subunits. Elsewhere, we will present evidence that these molecules are encoded in the *Qa-2* locus and that they vary in apparent molecular mass among a larger panel of other BALB.B CTL clones (unpublished data). It is not clear whether the 37- to 38-kDa components are involved in the function of these CTL.

Three laboratories have recently described what appears to be an Ag-recognition receptor on a mouse T-cell lymphoma of unknown function (5), on mouse T-helper-cell hybridomas (3), or on human cytotoxic T cells (4). In each case, the putative receptor seems to be a disulfide-linked heterodimer with subunit size within the 39–49 kDa range. We have shown here that the surface of mouse CTL clone 2C (and clones 1D and G4; data not shown) also has a disulfide-linked dimer (80–90 kDa) composed of 45-kDa subunits. The precipitation of this component by alloantisera to clone 2C indicates that, like the other T-cell clones, mouse CTL express a similar receptor. This possibility is obviously consistent with the ability of the clone-specific alloantisera to block cytolytic activity, but confirmation will require appropriate mAbs.

Antisera to idiotypes of Igs have long been available as clone-specific reagents for cells of B lineage (myelomas and others). The production of the corresponding antisera to T cells has until recently been frustrating, with occasional reports of success very difficult or impossible to reproduce. However, the recent availability of T-cell clones has drastically altered this situation and, by injecting such cloned cells in adequate numbers into appropriate recipients, we were able to elicit in virtually every immunized mouse alloantiserum that is clone specific. These antisera and the corresponding mAbs are likely to be of considerable value for elucidating the structure of T-cell surface molecules that are involved in recognition and destruction of target cells.

Note Added in Proof. In accord with the clone-specific alloantisera described above, we have recently identified a hybridoma, produced by fusing X63.653 cells with spleen cells from BALB/c mice immunized against clone 2C, that secretes a clone-specific mAb. The mAb inhibits 60–80% of the target cell lysis mediated by clone 2C but does not inhibit at all the lysis mediated by clone G4. This mAb is probably specific for the putative Ag-recognition receptor of clone 2C (Fig. 3*D*, arrow 1, and refs. 3–5).

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