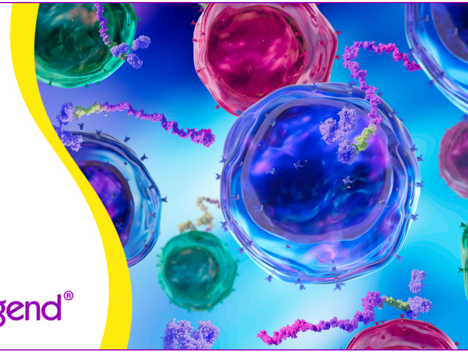
TotalSeq™ PhenoCyte
Ultra-high parameter, high-throughput, single-cell protein profiling
Learn more
BioLegend®



The Journal of Immunology
RESEARCH ARTICLE | DECEMBER 01 1980

IgG or IgM monoclonal antibodies reactive with different determinants on the molecular complex bearing Lyt 2 antigen block T cell-mediated cytolysis in the absence of complement. FREE

M Sarmiento; ... et. al

J Immunol (1980) 125 (6): 2665–2672.
https://doi.org/10.4049/jimmunol.125.6.2665

Related Content

High molecular weight lymphocyte surface proteins are structurally related and are expressed on different cell populations at different times during lymphocyte maturation and differentiation.

J Immunol (April,1982)

Analysis of the autoantibody response to fibrillarin in human disease and murine models of autoimmunity.

J Immunol (January,1995)

The presence of NK alloantigens on cloned cytotoxic T lymphocytes.

J Immunol (September,1983)

Downloaded from http://journals.aai.org/jimmunol/article-pdf/125/6/2665/1002182/2665.pdf by guest on 24 May 2025

0022-1767/80/1256-2665$02.00/0
THE JOURNAL OF IMMUNOLOGY
Copyright © 1980 by The Williams & Wilkins Co.
Vol. 125, No. 6, December 1980
Printed in U.S.A.

IgG OR IgM MONOCLONAL ANTIBODIES REACTIVE WITH DIFFERENT DETERMINANTS ON THE MOLECULAR COMPLEX BEARING LYT 2 ANTIGEN BLOCK T CELL-MEDIATED CYTOLYSIS IN THE ABSENCE OF COMPLEMENT[1]

MARION SARMIENTO,[2] ANDREW L. GLASEBROOK, AND FRANK W. FITCH

From the Department of Pathology and Committee on Immunology, University of Chicago, 950 East 59th Street Chicago, IL 60637

Five rat monoclonal antibodies have been derived that express specificities for determinants present on the molecular complex bearing the Lyt 2 antigen. SDS-polyacrylamide gel electrophoresis of ^{125}I-labeled polypeptides precipitated by each of these antibodies reveal 3 components (150,000, 75,000, and 33,000 daltons), and 2 components (44,000 and 33,000 daltons) when analyzed under nonreducing and reducing conditions, respectively. Two of these antibodies are IgG and are specific for the Lyt 2.2 determinant; the other 3 are IgM and react with determinants other than Lyt 2.2, which are nonpolymorphic. Each of the 5 antibodies can block the cytolytic activities of 5-day MLC cells or of cloned cytolytic T cells in the absence of C. Treatment of responding spleen cells with any of these antibodies and C inhibits the generation of cytolytic activity in MLC.

Antigenic determinants present on certain lymphocyte cell surface proteins can serve as markers for studying the relationship between the expression of these proteins and cellular maturation or function. Studies by Boyse *et al.* (1, 2) have shown that Lyt antigens are differentially expressed among thymus-dependent lymphocytes and that antigen-stimulated T cells can be classified according to cellular function on the basis of expression of these antigens at the cell surface. Thus, T cells that bear Lyt 2 or 3 antigens but not Lyt 1 usually express cytolytic or suppressor functions, whereas T cells that bear Lyt 1 but not Lyt 2 or 3 antigens express helper functions. The role of Lyt antigens in cellular maturation or function is not known. However, the observation that anti-Lyt 2 sera can block target cell lysis by cytolytic T cells in the absence of complement (C) suggests that the molecule bearing the Lyt 2 antigen may play a role in the cytolytic process (3, 4).

Two allelic forms of each of the Lyt antigens 1, 2, and 3 have been found in the mouse and have been designated Lyt 1.1 and 1.2, Lyt 2.1 and 2.2, and Lyt 3.1 and 3.2 (5). Genes controlling expression of Lyt 2 and 3 antigens are both present on chromosome 6, whereas the gene controlling Lyt 1 expression is located on chromosome 19 (5, 6). Genetic recombinations are known to occur between the Lyt 1 and Lyt 2 or 3 alleles but not between Lyt 2 and Lyt3 alleles (5).

The molecules bearing the Lyt 1, 2, or 3 determinants have been isolated by immune precipitation using antisera (7, 8). Polyacrylamide gel electrophoretic analyses of the precipitates has revealed that anti-Lyt 1 sera precipitates a molecular complex containing 2 polypeptides of apparent m.w. 67,000 and 87,000 (7). Other studies have shown that anti-Lyt 2 or anti-Lyt 3 sera can precipitate a polypeptide of 35,000 in m.w., although both of these antigens do not seem to be present on the same polypeptide (8, 9). More recently, analyses of immune precipitates obtained with anti-Lyt 2.2 sera have revealed that another polypeptide (apparent m.w. = 30,000) is precipitated together with the 35,000-dalton polypeptide, suggesting that both polypeptides are physically associated in a molecular complex in solutions containing nonionic detergent (10).

Cloned T cells provide a unique opportunity for studying the relationship between the cell surface proteins expressed by these cells and their functions. Such pure populations of lymphocytes facilitate selection of monoclonal antibodies that are specific for cell surface proteins expressed by functionally distinct T cells. T cells expressing either cytolytic or amplifier functions have been cloned in this laboratory from a unidirectional secondary C57BL/6 anti-DBA/2 mixed lymphocyte culture (MLC) and maintained in long-term culture (11, 12). Two cell lines, L3 and B18, have been found to express cytolytic activity specific for H-2D^d and H-2K^d gene products, respectively, and to bear the Lyt 2.2 antigen. Another cell line, L2, is noncytolytic and expresses amplifier activity but has no detectable levels of Lyt 2.2 In the present study, these cell lines were used to derive and characterize monoclonal antibodies that react with determinants present on the molecular complex bearing the Lyt 2 antigen. The experiments presented here indicate that monoclonal antibodies specific for the Lyt 2.2 antigen, as well as for other determinants present on the same molecular complex, can block cytolysis by both MLC cells and cloned cytolytic T cells in the absence of C. In addition, pretreatment of responding spleen cells with any of these monoclonal antibodies in the presence of C inhibits the generation of cytolytic activity in MLC.

MATERIALS AND METHODS

Animals. Lewis rats were obtained from Microbiological Associates. Congenic mutant mice C57BL/6 α (Lyt 1.1, 2.2) and C57BL/6 β (Lyt 1.2, 2.1) were kindly provided by H. Cantor. All other mice were obtained from The Jackson Laboratory.

Received for publication April 25, 1980.
Accepted for publication August 20, 1980.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by United States Public Health Service Grants AI-4197 and AI-14872.

[2] M.S. is a recipient of United States Public Health Service Postdoctoral Training Grant GM-07190.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/125/6/2665/1002182/2665.pdf by guest on 24 May 2025

Cells. Cell lines used to obtain rat-mouse hybrid cells were P3/X63/Ag8 (P3) (13) and SP2/0-Ag14 (SP2/0) (14). P-815 mastocytes (H-2^d, Thy 1.2, Lyt 2.1), syngeneic to DBA/2 mice, and AKR-A lymphoma cells (H-2^k, Thy 1.1, Lyt 2.2), syngeneic to AKR mice, were used as target cells in ^{51}Cr-release cytotoxicity assays. Conditions for the derivation and culturing of cloned cytolytic T cell lines L3 and B18 have been previously described (11, 12). Briefly, L3 cells were cultured for 5 days in the presence of C57BL/6 irradiated (1200 rads) spleen cells and Lewis concanavalin A supernatant factors (LCA SF)[3] before use in these studies. LCA SF was produced by culturing 25 × 10^6 Lewis rat spleen cells in 20 ml Dulbecco's minimum essential medium (DMEM; GIBCO) containing 2% fetal calf serum, 5 × 10^{-5} M 2-mercaptoethanol (2-Me)/10 mM morpholinopropanesulfonic acid (MOPS), penicillin/streptomycin, and 2.5 μg/ml concanavalin A (Pharmacia Fine Chemicals) in Falcon tissue culture flasks (No. 3013). The cells were cultured at 37°C with the flasks standing upright (i.e., 7.5 cm^2 growth area). After 48 hr, cells and debris were removed by centrifugation, and residual concanavalin A activity was adsorbed using Sephadex G-25 beads. The LCA SF was filtered through a sterile (0.22-μ) filtration unit and stored in 10-ml aliquots at −20°C for up to 1 mo.

Unidirectional primary MLC were generated as previously described (15). Upright Falcon tissue culture flasks (No. 3013) containing 25 × 10^6 each responding and stimulating cells, or flat-bottom tubes (13 mm^2) containing 5 × 10^6 each responding and stimulating cells were used in these studies.

Derivation of hybrid monoclonal antibodies and culture supernatants. A Lewis rat was immunized i.v. 3 times at 2-wk intervals with 15 × 10^6 L3 cloned cytolytic T cells. Three days after the third immunization, the rat spleen cells were fused with either P3 or SP2/0 cells using polyethylene glycol. Details of the technique used to obtain the rat-mouse hybrid cell lines using a C-mediated cytotoxicity assay for screening have been described (16). In order to obtain hybrid cell culture supernatants, hybrid cell lines were grown in spinner culture flasks with 250 ml DMEM containing 20% fetal calf serum, and penicillin/streptomycin. Cells and debris were removed by centrifugation, and culture supernatants were stored either frozen at −20°C or in the presence of 0.02% NaN_3 at 5°C.

T cell-mediated cytotoxicity, C-mediated cytotoxicity, and indirect immunofluorescence assays. T cell-mediated cytotoxicity was assayed using a ^{51}Cr-release cytotoxicity assay (15). Mouse thymocytes or spleen cells were tested for ability to be lysed by monoclonal antibodies using a C-mediated cytotoxicity assay (16). The monoclonal antibodies were also tested for ability to bind to thymocytes in an indirect immunofluorescent assay utilizing a fluorescein-coupled rabbit anti-rat immunoglobulin (Ig) antisera.

Treatment of responding spleen cells with antibody and C before placing in MLC. C57BL/6 and C3Heb/FeJ spleen cells were centrifuged in aliquots containing 5 × 10^6 cells, and each pellet was resuspended in 1 ml (undiluted) culture supernatant and placed at 5°C for 30 min. After centrifugation and removal of the culture supernatant by aspiration, the cells were resuspended in 1 ml agar-absorbed rabbit C at the appropriate dilution and incubated at 37°C for 45 min. The samples were pelleted and placed into 13-mm^2 flat-bottom tubes with 5 × 10^6 irradiated (2000 rads) DBA/2 spleen cells and incubated at 37°C. After 5 days, cultures were assayed for cytolytic activity using a ^{51}Cr-release cytotoxicity assay (15).

Assays for ability of monoclonal antibodies to block cytolytic activity of cloned cytolytic T cells or MLC cells in the absence of C. An adaptation of the short-term cytotoxicity assay described by MacDonald (17) was used. Briefly, cells from 6-day unidirectional C3HebFe/J anti-DBA/2 or C57BL/6 anti-DBA/2 MLC cells grown in upright Falcon tissue culture flasks (No. 3013) as previously described (15) were resuspended in 7.0 ml DMEM containing 5% fetal calf serum, 10 mM MOPS, and penicillin/streptomycin (assay media). Fifty microliters each of the undiluted and serial 1/3 diluted samples were plated in duplicate for each monoclonal antibody to be tested in round-bottom microtiter (Linbro No. IS-MRC-96) trays. Fifty microliters DMEM containing 20% fetal calf serum, or monoclonal antibody culture supernatants were added to each set of wells. The trays were placed at 5°C for 30 min, after which 5 × 10^3 P-815 target cells labeled with ^{51}Cr were added in 50 μl assay media to the appropriate wells. The trays were centrifuged at 200 × G for 1 min and incubated at 37°C for 30 min. Fifty microliters 0.04 M EDTA[3] in DMEM (adjusted to pH 7.2) were added to each well, and the trays were incubated at 37°C for an additional 2 hr. Afterward, the trays were centrifuged at 600 × G for 3 min, and 100 μl supernatant were removed from each well and counted in a gamma counter.

L3 cloned cytolytic T cells were used to test the ability of the monoclonal antibodies to block cytotoxicity in a similar manner as described above for MLC cells except that each well contained 1.25 × 10^5 L3 cells and 5 × 10^3 ^{51}Cr-labeled P-815 target cells, and the cells were incubated at 37°C for 5, 10, 20, 40 or 80 min before addition of EDTA.

Cell-surface radioiodination, methionine radiolabeling of monoclonal antibodies, Nonidet P-40 (NP-40) extraction of cells, and immune precipitation. These procedures have been described in detail elsewhere (18; Sarmiento, Loken, and Fitch, manuscript submitted for publication). Rabbit anti-rat Ig antisera or *Staphylococcus aureus* Cowan strain I (SaCI) (19) reagents were used in the immune precipitations.

SDS-polyacrylamide gel electrophoresis. Unless otherwise stated, all electrophoresis was performed on thin gels (10 cm x 14 cm x 0.75 mm) of 12% polyacrylamide cross-linked with diallyltartardiamide (DATD) under conditions previously described (16). Sample buffer for electrophoresis under reducing conditions consisted of 2% (w/v) SDS/5% (v/v) 2-Me/5% (w/v) sucrose/0.001% (w/v) bromophenol blue/0.05 M Tris-HCl, pH 7.0. 2-Me was omitted for analyses performed under nonreducing conditions. All samples were placed in a boiling water bath for 2 min. Sample volumes of 50 μl were analyzed per channel. Autoradiographs of fixed and dried gels containing ^{125}I-labeled samples were prepared with Dupont Cronex MRF 31 film and 2 Dupont Cronex Lightning Plus intensifying screens. Exposure was at −70°C for 3 to 14 days. Autoradiographs of gels containing ^{35}S-methionine labeled samples were exposed at room temperature for 12 hr using Dupont Cronex MRF 31 film.

Limited proteolysis analysis of immune precipitates. Limited proteolysis analysis was performed using a modification of the procedure described by Cleveland *et al.* (20). This method allows the direct comparison of peptides obtained from different proteins because the analyses are performed on an SDS-polyacrylamide slab gel. By using varying amounts of proteolytic enzyme, a peptide pattern can be obtained for each protein analyzed. Because the proteolysis is performed during electro-

[3] Abbreviations used in this paper: LCA SF, Lewis rat concanavalin A supernatant factor; 2-Me, 2-mercaptoethanol; SaCI, *Staphylococcus aureus* Cowan strain I; Sav8 protease, *Staphylococcus aureus* strain V8 protease; DMEM, Dulbecco's minimum essential medium; MOPS, morpholinopropane-sulfonic acid; EDTA, ethylenediaminetetraacetic acid; NP-40, Nonidet P-40; DATD, diallyltartardiamide.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/125/6/2665/1002182/2665.pdf by guest on 24 May 2025

phoresis, there is no loss of the sample in the analysis. Using this method of peptide analysis, immune precipitates obtained from NP-40 extracts of ^{125}I surface-labeled L3 cells using monoclonal antibody, were resuspended in 100 μl proteolysis sample buffer: 0.1% (w/v) SDS/1% (v/v) 2-Me/0.001 M EDTA/0.125 M Tris-HCl, pH 6.8. The samples were placed in a boiling water bath for 2 min. The analyses were performed on (15 cm x 14 cm x 0.75 mm) 15% polyacrylamide gels cross-linked with DATD. The stacker gel was 13.5 cm in length. Aliquots (10 μl) containing 2, 0.2, 0.04, or 0.008 μg of *Staphylococcus aureus* strain V8 (Sav8) protease or α-chymotrypsin (Miles Biochemicals) were placed into separate sample wells, and 50-μl aliquots of the immune precipitate sample were added to each. Electrophoresis was performed at 8 mAmps per gel, following which the gels were fixed, dried, and autoradiographed.

Reagents. SaCI was supplied by American Type Culture Collection. ^{51}Cr (100 to 400 mCi/mg), ^{35}S-methionine (600 to 1300 Ci/mmol, and carrier-free Na^{125}I (350 to 600 mCi/ml) are products of Amersham Corp. Reagents used for electrophoresis were supplied by Bio Rad. NP-40 was obtained from Gallard-Schlessinger. Rabbit anti-rat Ig was generated by repeatedly immunizing rabbits subcutaneously with immune complexes of rat Ig isolated from Ouchterlony gels in Freund's adjuvant. Lactoperoxidase and glucose oxidase were obtained from Sigma.

RESULTS

Derivation and characterization of monoclonal antibodies 2.43, 3.155, 3.168, 3.200, and 3.239. Hybrid rat-mouse cells were obtained by fusing spleen cells from a Lewis rat immunized with 15 × 10^6 L3 cells (cloned C57BL/6 cytolytic T cell expressing anti-H-2D^d specificity) with the mouse cell lines SP2/0 or P3. Wells containing hybrid cells were screened for ability to lyse L3 cells but not L2 cells (cloned C57BL/6 amplifier T cell), using a C-mediated cytotoxicity assay (16). Hybrid cells secreting antibody having this pattern of reactivity were cloned twice. Such monoclonal antibodies were tested for ability to block the cytolytic activity of L3 cells in a short-term ^{51}Cr-release microcytotoxicity assay in the absence of C. Five monoclonal antibodies, 2.43, 3.155, 3.168, 3.200, and 3.239 showed ability to block the killing of P-815 mastocytes by L3 cells and were further characterized for reactivity against the Lyt 2.2 antigen.

Culture supernatants from 2.43, 3.155, 3.168, 3.200, or 3.239 were tested for ability to react with thymocytes from several different mouse strains in a C-mediated cytotoxicity assay or by indirect immunofluorescence. As shown in Table I, monoclonal antibodies 3.155, 3.168, and 3.200 reacted with all thymocytes tested, regardless of Lyt phenotype, suggesting that these antibodies react with nonpolymorphic determinants. However, monoclonal antibodies 2.43 and 3.239 reacted only with cells bearing the Lyt 2.2 antigen. Monoclonal antibodies 83A and 15E, used as controls in these and other assays in this report, have been shown to react with Thy-1.2 antigen (Sarmiento, Loken, and Fitch, manuscript submitted for publication).

Figure 1 shows results obtained with each of the monoclonal antibodies in a ^{51}Cr-release C-mediated cytotoxicity assay using thymocytes or spleen cells from congenic C57BL/6 mutant mice, α (Lyt 1.1, 2.2), or β (Lyt 1.2, 2.1) and normal C57BL/6 mice (Lyt 1.2, 2.2). All 5 monoclonal antibodies showed cytotoxicity against C57BL/6 α and normal C57BL/6 thymocytes (Fig. 1, panels a and b). However, only 3.168, 3.200, and 3.155 reacted with C57BL/6 β thymocytes (Fig. 1, panel b). All 5 monoclonal antibodies were able to lyse approximately 85% and 15% of normal C57BL/6 thymocytes and spleen cells, respectively (panels c and d). The reactivity with spleen cells was lost if the cells were previously treated with anti-Thy1.2 monoclonal antibody and C, suggesting that the monoclonal antibodies react with T (Thy-1-positive) cells. These data also indicate that monoclonal antibodies 2.43 and 3.239 are specific for cells bearing the Lyt 2.2 antigen.

In order to determine the Ig classes of each of the monoclonal antibodies, the hybrid cells were cultured for several hours in the presence of ^{35}S-methionine, and aliquots of the culture supernatants obtained from each were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. Figure 2 shows the major polypeptide bands synthesized and secreted by each of the hybrid cells during the labeling period. Hybrid cells 3.239, 3.155, and 3.168 were obtained by fusion with the P3 myeloma, and consequently, each secretes a γ heavy chain polypeptide (apparent m.w. = 50,000) and a light chain polypeptide (apparent m.w. = 25,000), which correspond in electrophoretic mobility to those secreted by the P3 myeloma. Hybrid cell 3.239 produces an additional γ-chain, and hybrid cells 3.155 and 3.168 synthesize other heavy chain polypeptides that resemble μ-chains in electrophoretic mobility (apparent m.w. = 75,000). Hybrid cell 3.200, which was also fused with P3, secretes 2 light chains, although only 1 heavy chain polypeptide (μ) is produced by this cell, suggesting that the P3 γ-chain polypeptide is either no longer synthesized or secreted by this cell. Hybrid cell line 2.43 was fused with the nonsecretor cell line, SP2/0, and consequently shows significant incorporation of label into single heavy γ and light chain poly-

TABLE I

Reactivity of monoclonal antibodies 2.43, 3.155, 3.168, 3.200, 3.239, 15E, and P3 with thymocytes of different genotypes[a]

Thymus Cells	Genotype						C Cytotoxicity (a)						Immunofluorescence (b)				
	H-2	Ig	Lyt 1	Lyt 2	Lyt 3	Lyt 6	43	155	168	200	239	83A	43	155	239	15E	P3
A/J	a	e	2	2	2		+	+	+	+	+	+					
AKR/J	k	d	2	1	1	2	−	+	+	+	−	−	−	+	−	−	−
BALB/c	d	a	2	2	2	1	+	+	+	+	+	+	+	+	+	+	−
CAL20	d	e	2	2	2		+	+	+	+	+	+					
CBA/J	k	a	1	1	2	1	−	+	+	+	−	+	−	+	−	+	−
CB20	d	b	2	2	2		+	+	+	+	+	+					
C57BL/6	b	b	2	2	2	2	+	+	+	+	+	+	+	+	+	+	−
C3Heb/FeJ	k	a	1	1	2		−	+	+	+	−	+	−	+	−	+	−
DBA/2	d	c	1	1	2	2	−	+	+	+	−	+					
SJL	s	b	2	2	2		+	+	+	+	+	+					

[a] Monoclonal antibodies were tested for ability to react with thymocytes from various mouse strains in a C-mediated cytotoxicity assay (a) or by indirect immunofluorescence assay (b). The genotypes of the mouse strains tested are indicated.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/125/6/2665/1002182/2665.pdf by guest on 24 May 2025

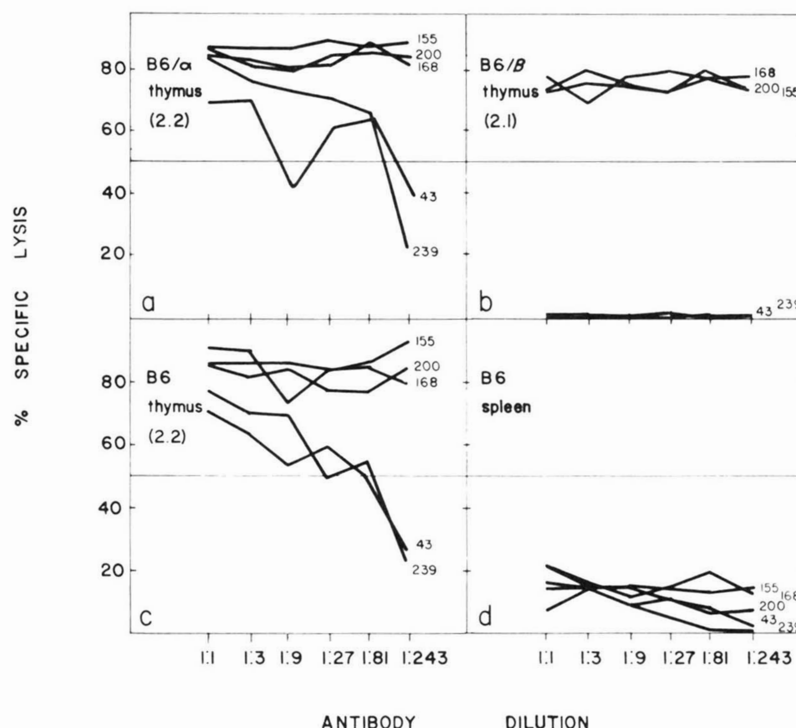


Figure 1. C-mediated cytotoxicity of thymocytes and spleen cells by monoclonal antibodies. Serial dilutions of monoclonal antibodies 2.43, 3.155, 3.168, 3.200, and 3.239 were tested for ability to lyse ^{51}Cr-labeled thymocytes or spleen cells from C57BL/6 α (Lyt 1.1, 2.2), C57BL/6 β (Lyt 1.2, 2.1), or C56BL/6 (Lyt 1.2, 2.2) mice in a C-mediated cytotoxicity assay. Spontaneous lysis of target cells was 5–10%.

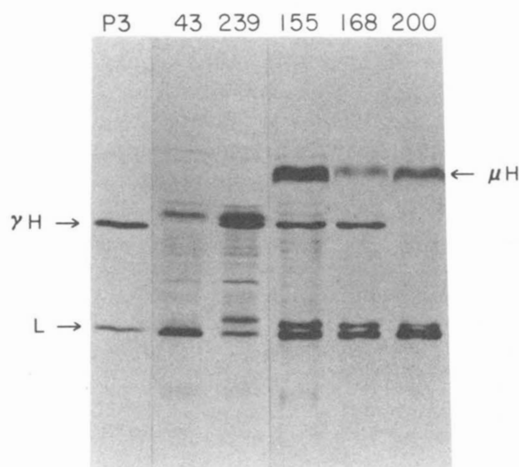


Figure 2. Ig polypeptides secreted by hybrid cells 2.43, 3.155, 3.168, 3.200, and 3.239. Hybrid cells or P3 parental cells were cultured in the presence of ^{35}S-methionine and the culture supernatants were analyzed on SDS-polyacrylamide gels followed by autoradiography. *H* indicates positions of heavy chain γ or μ polypeptides; *L* indicates positions of light chain polypeptides.

peptides. Results obtained from gel diffusion (Ouchterlony) studies with each of these monoclonal antibodies using Ig class-specific antisera (kindly provided by H. Bazin) also indicate that hybrid cell 2.43 secretes a rat IgG2b antibody; the IgG subclass of 3.239 was not determined.

Ability of monoclonal antibodies 2.43, 3.155, 3.168, 3.200, and 3.239 to inhibit the generation of cytolytic activity in MLC. Aliquots of spleen cells (5 × 10^{6} cells) from C3Heb/FeJ and C57BL/6 mice were treated with 1 ml undiluted culture supernatants or medium in the presence of rabbit C and were cultured together with 5 × 10^{6} irradiated DBA/2 cells at 37°C. Five days later, cells from each culture were assayed for ability to lyse P-815 mastocyte target cells in a ^{51}Cr-release cytotoxicity assay. The data shown in Figure 3, panel b, indicate that pretreatment with monoclonal antibodies 2.43, 3.155, 3.168, 3.200, and 3.239, and anti-Thy-1.2 monoclonal antibody (15E) significantly reduced the cytolytic activity generated by C57BL/6 responding cells below that observed by pretreatment with P3 culture supernatant, or media. The results in Figure 3, panel a, indicate that although pretreatment with 3.155, 3.168, 3.200, or 15E reduced cytolytic activity generated by responding C3Heb/FeJ spleen cells below control levels, monoclonal antibodies 2.43 and 3.239 had no detectable effect at the concentration used (undiluted culture supernatant) or at 10-fold concentration (data not shown). It has also been observed that treatment of cells from unidirectional C57BL/6 anti-DBA/2 5-day primary MLC with monoclonal antibodies 2.43, 3.155, 3.168, 3.200, or 3.239 and C abolishes the ability of these cells to lyse P-815 target cells. However, ability to lyse P-815 targets by cells from unidirectional C3Heb/Fej anti-DBA/2 5-day primary MLC is lost by treatment in the presence of C with 3.155, 3.168, and 3.200 but *not* with 2.43 and 3.239 (data not shown). In contrast to what was found with 3.155, 3.168, or 3.200, fluorescent antibody assays have also failed to show reactivity by either 2.43 or 3.239 with C3H, AKR, or CBA thymocytes (see Table I). These results are consistent with the finding that monoclonal antibodies 3.155, 3.168, and 3.200 react with thymocytes or T cells regardless of Lyt 2 phenotype, whereas 2.43 and 3.239 react with cells that express the Lyt 2.2 antigen (Table I).

Ability of monoclonal antibodies 2.43, 3.155, 3.168, 3.200, and 3.239 to block cytolytic activity of 5-day MLC or of cloned cytolytic T cells in the absence of C. Five-day unidirectional C57BL/6 anti-DBA/2 or C3Heb/FeJ anti-DBA/2 MLC cells were tested in a short-term ^{51}Cr-release cytotoxicity assay for ability to lyse P-815 targets in the presence of monoclonal antibodies 2.43, 3.155, 3.168, 3.200, 3.239, 15E, or controls P3 or media. The data shown in Figure 4, panel a, indicate that 3.155, 3.168, or 3.200 significantly reduced cytolysis of P-815 targets by C3Heb/FeJ anti-DBA/2 MLC cells, whereas 2.43 and 3.239 had no effect. However, all 5 monoclonal antibodies markedly blocked the killing of P-815 target cells by C57BL/6 anti-DBA/2 MLC cells (Fig. 4, panel b). The cloned T cell line L3 has been shown to be of C57BL/6 origin and expresses Lyt 2.2 antigen on the cell surface (11, 12). Monoclonal antibodies 2.43,

Downloaded from http://journals.aai.org/jimmunol/article-pdf/125/6/2665/1002182/2665.pdf by guest on 24 May 2025

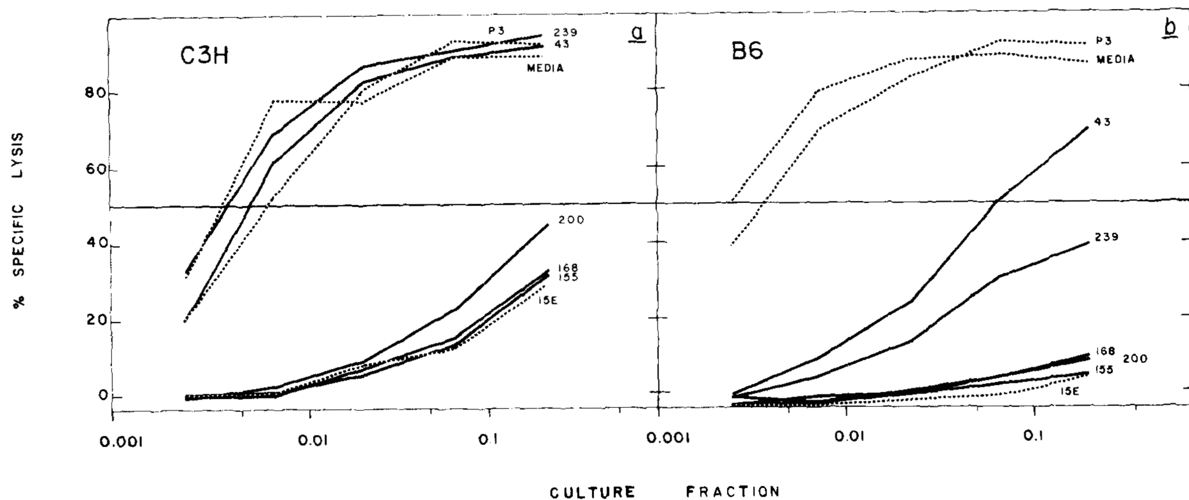


Figure 3. Inhibition of the generation of cytotoxicity in MLC by pretreatment of responding spleen cells with monoclonal antibodies and C. C3Heb/FeJ (*panel a*) or C56BL/6 (*panel b*) spleen cells were treated with monoclonal antibodies 2.43, 3.155, 3.168, 3.200, 3.239, 15E, P3, or media, and C, and cultured with irradiated DBA/2 spleen cells for 5 days. Cultures were assayed for cytolytic activity against P-815 target cells by using a ^{51}Cr-release assay. Spontaneous lysis was 4–7%.

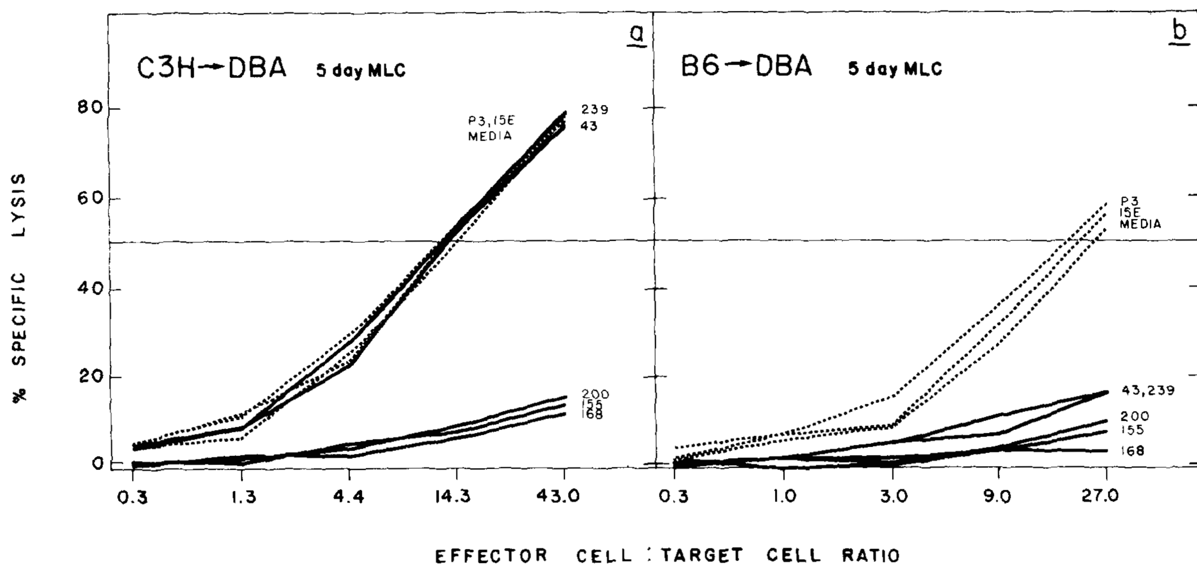


Figure 4. Blocking of cytotoxicity of 5 day MLC by monoclonal antibodies 2.43, 3.155, 3.168, 3.200, and 3.239 in the absence of C. Five day C3Heb/FeJ anti-DBA/2 or C57BL/6 anti-DBA/2 MLC cells were assayed in a short-term ^{51}Cr-release cytotoxicity assay at various effector: target cell ratios for ability to lyse P-815 target cells in the presence of monoclonal antibodies 2.43, 3.155, 3.168, 3.200, 3.239, 15E, or P3, or media.

3.200, and 3.239 were tested for ability to block lysis of P-815 target cells by L3 cells in a short-term ^{51}Cr-release cytotoxicity assay. As shown in Table II, each of these monoclonal antibodies prevented lysis of P-815 targets in the absence of C when the cells were incubated for up to 80 min before addition of EDTA. Cells treated in this manner and incubated for up to 24 hr were capable of excluding trypan blue dye.

Monoclonal antibodies 2.43, 3.155, 3.168, 3.200, and 3.239 react with the same molecular complex. The data obtained from the functional assays indicate that although monoclonal antibodies 2.43, 3.155, 3.168, 3.200, or 3.239 can each block cytolytic activity, only 2.43 and 3.239 are specific for cells bearing Lyt 2.2. In order to identify the molecular targets of 3.155, 3.168, and 3.239, immune precipitates were obtained using these antibodies. L3 cells were surface labeled with ^{125}I, and an NP-40 extract was obtained. Culture supernatants from 2.43, 3.155, 3.168, 3.200, 3.239, or P3 were mixed with aliquots of NP-40 extract, and precipitates were obtained using a rabbit anti-rat Ig sera. The precipitates were dissolved in sample buffer for electrophoresis and were analyzed on 12% SDS-polyacrylamide gels cross-linked with DATD. Electrophoresis was performed in the presence (reducing conditions) or absence (non-reducing conditions) of 2-Me. The autoradiographs presented in Figure 5 show that all five monoclonal antibodies precipitate the same set of molecules. Under reducing conditions (top panel), the major band has a mobility of approximately 44,000 daltons. A faint band (apparent m.w. = 33,000) is also present in each of these precipitates. Under nonreducing conditions (lower panel), no band is visible at 44,000 daltons, although most of the radioactive label is detectable in a component of approximately 75,000 daltons. The band at 33,000 daltons is also present under these conditions, although barely visible in the autoradiograph. In addition, a novel band is detectable at approximately 150,000 daltons. Analyses performed on a 5% polyacrylamide gel revealed that there are no polypeptide bands greater than 150,000 daltons present under nonreducing conditions. The antibody secreted by P3 myeloma (control) failed to precipitate any labeled polypeptides. It should be noted that the acrylamide

Downloaded from http://journals.aai.org/jimmunol/article-pdf/125/6/2665/1002182/2665.pdf by guest on 24 May 2025

TABLE II

Blocking of cytolytic activity of a mouse T lymphocyte clone by monoclonal antibodies 2.43, 3.200, 3.239, or P3[a]

Incubation Time (Min)	% Inhibition (E:T = 25:1)			
	43	200	239	P3
5	100	100	95	0
10	100	100	87	0
20	100	100	87	0
40	100	100	80	0
80	84	83	88	0

[a] L3 cells were treated with monoclonal antibodies for 30 min at 5°C. The cells were then assayed for cytotoxicity against P-815 target cells at 25:1 effector:target (E:T) cell ratio in a short-term ^{51}Cr-release assay (17). EDTA was added after 5, 10, 20, 40, or 80 min incubation with P-815 targets at 37°C. Amount of ^{51}Cr released into the supernatant was determined at 2 hr (total) incubation. Spontaneous lysis of P-815 target cells was 7%; specific lysis by L3 cells in the presence of media was 80%.

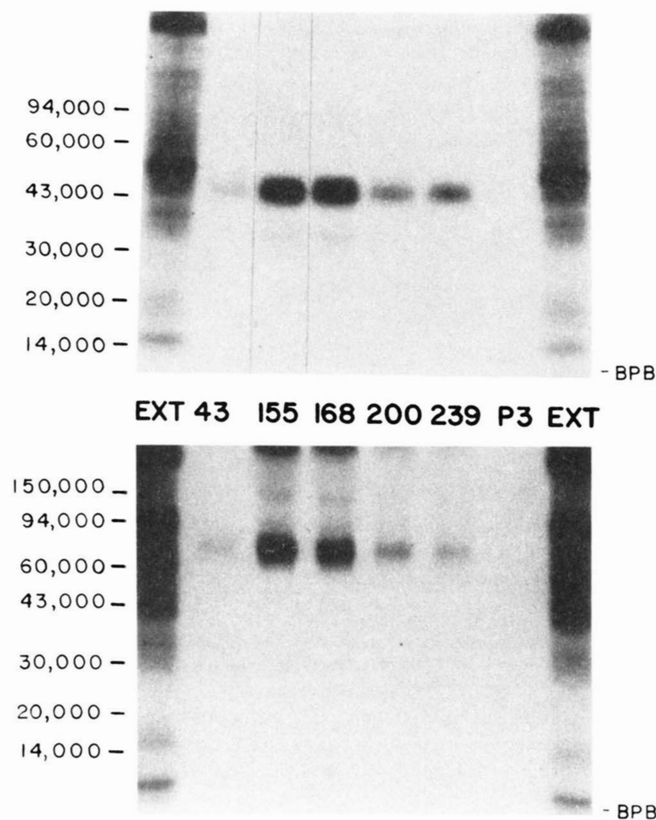


Figure 5. Electrophoretic analysis in the presence or absence of 2-Me of polypeptides precipitated by monoclonal antibodies 2.43, 3.155, 3.168, 3.200, and 3.239. Monoclonal antibodies 2.43, 3.155, 3.168, 3.200, 3.239, or P3 were used to obtain immune precipitates from an NP-40 extract of L3 cells previously surface-labeled with ^{125}I. The precipitates were analyzed on 12% SDS-polyacrylamide slab gels under reducing (+ 2-Me) or nonreducing (− 2-Me) conditions, and the gels were autoradiographed. BPB indicates position of bromphenol blue dye.

gels used in these studies are cross-linked with DATD instead of bis and give somewhat different m.w. estimates than previously reported for polypeptides precipitated with anti-Lyt 2.2 sera (8, 10).

In order to further demonstrate the similarity of the molecules precipitated by the monoclonal antibodies, a comparison was made of the peptide band patterns obtained by digestion of precipitated target molecules with SaV8 protease or α-chymotrypsin, using the method of Cleveland *et al.* (20). Monoclonal antibodies 3.239 (anti-Lyt 2.2) and 3.155 (nonpolymorphic) were used to obtain immune precipitates from NP-40 extracts of ^{125}I-labeled L3 cells. Each precipitate was dissolved in proteolysis buffer, placed in a boiling water bath for 2 min, and equal volumes (50 μl) were placed in multiple sample wells on 15% acrylamide slab gels. Varying amounts of SaV8 protease or chymotrypsin were added to each well, and the samples were electrophoresed. The autoradiographs shown in Figure 6 reveal that the polypeptides precipitated by either 3.239 or 3.155 produced similar patterns of digestion when treated with each enzyme. Treatment with SaV8 protease yielded at least 2 novel peptide fragments at the highest amount of enzyme used. However, digestion with chymotrypsin resulted in approximately 6 well-resolved peptides. The small number of peptides generated by SaV8 protease suggests that the polypeptides precipitated by these antibodies may only have a few readily accessible glutamoyl bonds. The similarity in the effects of either SaV8 protease or chymotrypsin on 3.239 or 3.155 precipitates together with the similarities observed in electrophoretic mobilities of precipitated polypeptides analyzed under reducing or nonreducing conditions (see Fig. 5) indicate that although 3.239 and 3.155 recognize different determinants, the same molecules are precipitated by either antibody. It should be noted that in Figure 6, some labeled material is detectable above the 44,000-dalton band. This appears to be due to incomplete reduction and/or aggregation of the smaller polypeptides in the presence of low amounts of 2-Me and SDS in the sample buffer. When separate aliquots of the samples used in Figure 6 were adjusted to 5% (v/v) 2-Me and 2% (w/v) SDS and were analyzed on acrylamide gels, only the 44,000- and 33,000-dalton polypeptides were evident (data not shown).

Collectively, these data demonstrate that each of these 5 monoclonal antibodies blocks T cell-mediated cytolysis by bind-

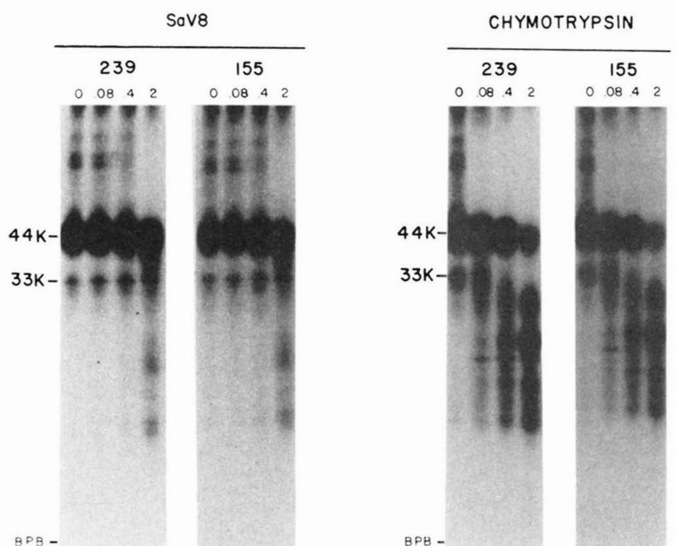


Figure 6. Limited proteolysis analysis of polypeptides precipitated by monoclonal antibodies 3.239 and 3.155. Immune precipitates obtained from an NP-40 extract of ^{125}I surface-labeled L3 cells by using monoclonal antibodies 3.239 (anti-Lyt 2.2) or 3.155 (non-polymorphic) were digested with 0 μg, 0.08 μg, 0.4 μg, or 2 μg of SaV8 protease or α-chymotrypsin during SDS-polyacrylamide electrophoresis in the presence of 2-Me and SDS according to the method of Cleveland *et al.* (20). Labeled polypeptides and peptide fragments were exposed by autoradiography. The autoradiographs were overexposed in order to reveal minor bands. 44K and 33K indicate the positions of the intact 44,000 dalton and 33,000 dalton components, respectively.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/125/6/2665/1002182/2665.pdf by guest on 24 May 2025

ing to a molecular complex on the surface of the cytolytic T cell, which contains at least 2 polypeptides of 44,000 daltons and 33,000 daltons in size. Two of the antibodies have specificity for the Lyt 2.2 determinant, and the remaining 3 react with other, nonpolymorphic determinants present on the same complex, indicating that antibody-mediated blocking of cytolysis is not restricted to binding to the Lyt 2.2 determinant alone.

Cell surface polypeptides isolated by anti-Lyt 2.2 monoclonal antibody are less heterogeneous than those isolated by anti-Thy-1.2 monoclonal antibody. Recent findings in this laboratory have shown that cell surface molecules bearing the Thy-1.2 determinant can exhibit different electrophoretic band patterns when isolated from different cells (Sarmiento, Loken, and Fitch, manuscript submitted for publication). In order to determine whether the polypeptides of the molecular complex bearing the Lyt 2 determinant also exhibit heterogeneity at the cell surface, immune precipitates were obtained using monoclonal antibodies 3.239 (anti-Lyt 2.2) and 15E (anti-Thy-1.2) from NP-40 extracts of 2 ^{125}I-surface-labeled syngeneic (C57BL/6) cytolytic T cell clones, L3 and B18. The autoradiographs in Figure 7 show that polypeptides isolated by 15E from L3 cells produce a different electrophoretic band pattern from those isolated by 15E from B18 cells. However, the electrophoretic mobilities of the 44,000-dalton and 33,000-dalton polypeptides isolated by 3.239 from L3 or B18 cells are very similar. Similar electrophoretic band patterns have been obtained whether the

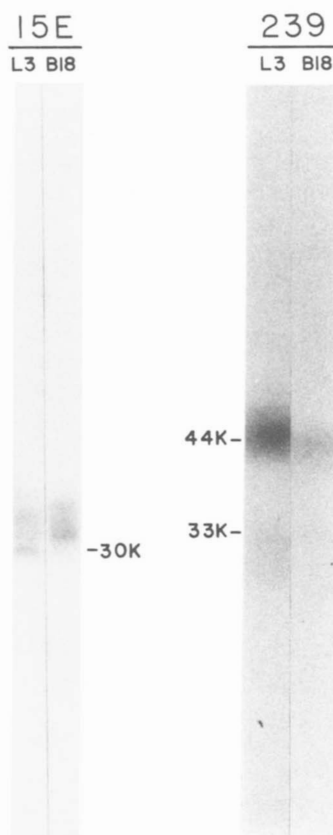


Figure 7. SDS-polyacrylamide electrophoretic comparison of cell surface polypeptides precipitated from L3 and B18 cells by anti-Thy-1.2 or anti-Lyt 2.2 monoclonal antibodies. Monoclonal antibodies 15E (anti-Thy-1.2) or 3.239 (anti-Lyt 2.2) were used to obtain immune precipitates from NP-40 extracts of L3 and B18 cells previously surface-labeled with ^{125}I. Precipitated polypeptides were analyzed on 12% polyacrylamide gels in the presence of 2-Me followed by autoradiography.

analyses were performed using T cell clones cultured for 1 mo or for 1½ yr after isolation. These data indicate that, unlike the molecule bearing the Thy-1 determinant, the cell surface polypeptides precipitated by anti-Lyt 2.2 antibody do not exhibit marked differences in electrophoretic band pattern when isolated from 2 different but syngeneic cytolytic T cell clones.

DISCUSSION

Cloned murine T lymphocytes have been used to derive 5 monoclonal rat antibodies that react with the molecular complex bearing the Lyt 2 antigen. By screening against functionally distinct T cell clones, (i.e., cytolytic *vs* amplifier), hybrid cells secreting antibody reactive with 1 cell type but not the other could be rapidly selected. The cytolytic T cell clones were also useful for testing monoclonal antibodies for ability to block cytotoxicity, as has been found with anti-Lyt 2.2 sera (3). Two of the antibodies selected are IgG and have specificity for Lyt 2.2; the other 3 are IgM and react with nonpolymorphic determinants present on the same molecular complex.

Earlier studies (10) using SDS-polyacrylamide gel electrophoresis to analyze precipitates obtained with anti-Lyt 2 antibodies demonstrated 2 polypeptides (apparent m.w. = 35,000 and 30,000) on reducing gels and a band of approximately 65,000 daltons on nonreducing gels. These data suggested that the 65,000-dalton band contains dimers of the 35,000-dalton and 30,000-dalton polypeptides, which are held together by disulfide bonds. Using gels cross-linked with DATD instead of bis, the 35,000- and 30,000-dalton components appear as bands of 44,000 and 33,000 daltons in size; the 65,000-dalton polypeptide appears as a band of 75,000 daltons. In the present studies, faint bands are also detectable at 150,000 daltons and 33,000 daltons under nonreducing electrophoretic conditions.

The study comparing cell surface components precipitated with anti-Lyt 2.2 or anti-Thy-1.2 indicates that between 2 syngeneic cytolytic T cell clones, molecules recognized by anti-Lyt 2.2 antibody have very similar electrophoretic band patterns, whereas polypeptides bearing the Thy-1 determinant show marked differences. The basis for the structural diversity of cell surface polypeptides bearing the Thy-1.2 determinant isolated from different cell types is not known. However, it has been shown that much of the electrophoretic heterogeneity of molecules bearing the Thy-1 antigen is probably due to differences in the glycosylation on a common polypeptide chain (21). The data presented here suggest that the 44,000-dalton and 33,000-dalton polypeptides of the molecular complex bearing the Lyt 2 antigen may not be as differentially glycosylated.

We have observed that pretreatment of responding spleen cells with any of the 5 monoclonal antibodies in the presence of C significantly reduces the levels of cytolytic activity generated in MLC. These data suggest that a noncytolytic T cell population, which expresses at the surface, the molecular complex bearing the Lyt 2 determinant, is required for the generation of cytotoxicity in MLC. Cytotoxicity by MLC cells has previously been shown to be insensitive to treatment with a variety of antibodies in the absence of C (22), although more recently it has been found that cytolytic activity can be blocked using anti-Lyt 2 sera (3). All 5 monoclonal antibodies in the present study can block T cell-mediated cytolytic activity of either MLC or cloned cytolytic T cells in the absence of C, indicating that blocking of cytolysis can also be obtained by binding antibody to determinants other than Lyt 2 and supporting the notion that the molecular complex bearing the Lyt 2 determinant may play a functional role in the cytolytic mechanism.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/125/6/2665/1002182/2665.pdf by guest on 24 May 2025

Acknowledgments. We thank H. Cantor for providing the congenic mutant C57BL/6 mice, and H. Bazin for the kind gift of Ig class-specific sera. The excellent technical assistance of L. Decker is acknowledged.

REFERENCES

1. Boyse, E. A., M. Miyazawa, T. Aoki, and L. J. Old. 1968. Ly-A and Ly-B: two systems of lymphocyte isoantigens in the mouse. Proc. R. Soc. Lond. (Biol.) 170:175.
2. Boyse, E. A., K. Itakura, E. Stockert, C. A. Iritani, and M. Miura. 1971. Lyc: a third locus specifying alloantigens expressed only on thymocytes and lymphocytes. Transplantation 11:351
3. Nakayama, E., H. Shiku, E. Stockert, H. F. Oettgen, and L. J. Old. 1979. Cytotoxic T cells: lyt phenotype and blocking of killing activity by Lyt antisera. Proc. Natl. Acad. Sci. 76:1977.
4. Shinohara, N., and D. H. Sachs. 1979. Mouse alloantibodies capable of blocking cytotoxic T-cell function. I. Relationship between the antigen reactive with blocking antibodies and the Lyt-2 locus. J. Exp. Med. 150:432.
5. Itakura, K., J. J. Hutton, E. A. Boyse, and L. J. Old. 1972. Genetic linkage relationships of loci specifying differentiation alloantigens in the mouse. Transplantation 13:239.
6. Itakura, K., J. J. Hutton, E. A. Boyse, and L. J. Old. 1974. Linkage groups of the theta and Ly-A loci. Nature (New Biol.) 230:126.
7. Durda, P. J., C. Shapiro, and P. D. Gottlieb. 1978. Partial molecular characterization of the Ly-1 alloantigen on mouse thymocytes. J. Immunol. 120:53.
8. Durda, P. J., and P. D. Gottlieb. 1976. The Ly-3 antigens on mouse thymocytes: immune precipitation and molecular weight characterization. J. Exp. Med. 144:476.
9. Durda, P. J., and P. D. Gottlieb. 1978. Sequential precipitation of mouse thymocyte extracts with anti-Lyt-2 and anti-Lyt-3 sera. I. Lyt 2.1 and Lyt 3.1 antigenic determinants reside on separable molecular species. J. Immunol. 121:983.
10. Ledbetter, J. A., and L. A. Herzenberg. 1979. Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol. Rev. 47:63.
11. Glasebrook, A. L., and F. W. Fitch. 1979. T-cell lines which cooperate in the generation of specific cytolytic activity. Nature 278:171.
12. Glasebrook, A. L., and F. W. Fitch. 1980. Alloreactive cloned T cell lines. I. Interactions between cloned amplifier and cytolytic T cell lines. J. Exp. Med. 151:876.
13. Williams, A. F., G. Galfre, and C. Milstein. 1977. Analysis of cell surfaces by xenogeneic myeloma hybrid antibodies: differentiation antigens on rat lymphocytes. Cell 12:663.
14. Shulman, M., C. D. Wilde, and G. Kohler. 1978. A better cell line for making hybridomas secreting specific antibodies. Nature 276: 269.
15. Tartof, D., and F. W. Fitch. 1977. Immunologically specific cytolytic activity induced in long-term mixed leukocyte culture cells by concanavalin A. J. Immunol. 118:35.
16. McKearn, T. J., F. W. Fitch, D. E. Smilek, M. Sarmiento, and F. P. Stuart. 1979. Properties of rat anti-MHC antibodies produced by cloned rat-mouse hybridomas. Immunol. Rev. 47:91.
17. MacDonald, H. R. 1975. Early detection of potentially lethal events in T cell-mediated cytolysis. Eur. J. Immunol. 5:251.
18. Sarmiento, M., A. L. Glasebrook, and F. W. Fitch. 1980. Cell surface polypeptides of murine T-cell clones expressing cytolytic or amplifier activities. Proc. Natl. Acad. Sci. 77:1111.
19. Cullen, S. E., and B. D. Schwartz. 1976. An improved method for isolation of H-2 and Ia alloantigens with immunoprecipitation induced by protein A-bearing staphylococci. J. Immunol. 117:136.
20. Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and V. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. 252:1102.
21. Campbell, D. G., A. F. Williams, P. M. Bayley, and K. B. M. Reid. 1979. Structural similarities between Thy-1 antigen from rat brain and immunoglobulin. Nature 282:341.
22. Kimura, A. K., and H. Wigzell. 1977. Cytotoxic T lymphocyte membrane components: an analysis of structures related to function. Contemp. Top. Mol. Immunol. 6:209.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/125/6/2665/1002182/2665.pdf by guest on 24 May 2025