

## A CLONE-SPECIFIC MONOCLONAL ANTIBODY THAT INHIBITS CYTOLYSIS OF A CYTOLYTIC T CELL CLONE\*

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Cytolytic T lymphocytes (CTL)<sup>1</sup> are thought to be important effector cells in immunity to virus infection and in allograft rejection. The antigenic specificity of cytotoxicity mediated by CTL has now been documented using cloned CTL populations (1-3). This specificity is mediated through antigen-specific receptors on the cell surface. The receptor specificities of alloreactive CTL and their precursors represent phenotypic markers which can be used to distinguish clonally distributed subpopulations of T lymphocytes that otherwise have very similar cell surface characteristics (4, 5). Under the appropriate culture conditions, this antigen specificity has been shown to be a stable phenotypic property of cloned T lymphocytes (5).

Antisera reactive with specific populations of cytolytic T lymphocytes have been reported. These antisera were obtained by immunizing mice with heterogeneous populations of cells derived from alloreactive mixed lymphocyte cultures (MLC) (6), or activated spleen cells reactive with trinitrophenyl (TNP)-modified (7) or virus-modified (8) target cells. Given the extremely diverse repertoire of CTL (2, 4), it seems unlikely that these antisera were reactive with individual idiotypes expressed by CTL. Recent studies in this laboratory have shown that cloned cytolytic T lymphocytes are particularly suited as a source of homogeneous cells for use in functional assays designed to identify monoclonal antibodies reactive with cell surface structures associated with immunological functions (9). This approach has been used successfully to derive xenogeneic monoclonal antibodies reactive with the L<sub>yt</sub>-2,3 (10) and LFA-1 (9, 11) cell surface molecules. In the present study, this approach has been applied in the screening of hybridoma antibodies potentially directed against antigen recognition structures on the cloned alloreactive CTL line, L3 (12). Here we report the production of a monoclonal antibody, designated 384.5, that inhibits the lytic activity of the L3 CTL clone. This antibody is distinguished from previously reported antibodies that inhibit cytolytic activity in that the inhibition is specific for the L3 cytolytic clone. The clone-specific nature of the reactivity of this antibody has been

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<sup>1</sup> *Abbreviations used in this paper:* AGH, agammaglobulinemic horse serum; CTL, cytotoxic T lymphocyte; Con A, concanavalin A; DME, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; FCS, fetal calf serum; MHC, major histocompatibility complex; MLC, mixed lymphocyte culture; PHA, phytohemagglutinin; TNP, trinitrophenyl.

confirmed by immunofluorescent labeling and analysis by immunofluorescence flow cytometry.

### Materials and Methods

*Experimental Animals.* Adult female mice of strains C57BL/6, CBA/J, DBA/2, and C57BL/6 × DBA/2 F<sub>1</sub> (BDF<sub>1</sub>) were obtained from The Jackson Laboratory, Bar Harbor, ME, or Laboratory Supply Co., Indianapolis, IN. Adult mice from strains B10.D2 and B10.A(5R)/Sgn were obtained as the progeny of breeding pairs obtained from The Jackson Laboratory and maintained at the University of Chicago.

*Cloned T Lymphocytes.* CTL clones L3 and B18 are of C57BL/6 anti-DBA/2 origin (12). L3 specifically lyses target cells that express surface antigens controlled by genes located in the right half of the *H-2<sup>d</sup>* haplotype of the major histocompatibility complex (MHC) (5). B18 is specific for target cells expressing antigens controlled by the left half of the MHC, probably *H-2K<sup>d</sup>* (5). The CTL clones T18 and T38 were derived from primary C57BL/10 (B10) anti-TNP-B10 MLC (13). Clone T18 specifically lyses TNP-modified target cells expressing *H-2D<sup>b</sup>* antigen. Clone T38 specifically lyses TNP-modified cells that bear *H-2D<sup>b,d,s</sup>* antigens. The CTL clones L3, B18, T18, and T38 express Lyt-2:2 antigen on their surface.

The maintenance of the CTL clones L3 and B18 has been described previously (3). Briefly, L3 and B18 cells were maintained by weekly restimulation with irradiated alloantigen and secondary MLC supernatant in Dulbecco's modified Eagle's medium (DME) (H-21; Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) containing 2% fetal calf serum (FCS) (KC Biological, Inc., Lenexa, KS), 100 U/ml penicillin, 100 µg/ml streptomycin, and additional (14) amino acids. Each well (Linbro 24-well plate, 76-033-05; Linbro Chemical Co., Hamden, CT) contained  $2 \times 10^4$  L3 or B18 cells,  $6.5 \times 10^6$  irradiated (1,400 rad) (model 143 cesium irradiator: JLS Shepherd & Associates, Glendale, CA) DBA/2 spleen cells, and 33% supernatant from secondary MLC in a final volume of 1.6 ml. Culture plates were then incubated in a 37°C 5% CO<sub>2</sub> humidified incubator.

*Antibodies.* Monoclonal antibodies 2.43 (anti-Lyt-2.2), 3.155 (anti-Lyt-2), 15E (anti-Thy-1.2), and 83A (anti-Thy-1.2) have been described (10, 15). Fluorescein isothiocyanate-coupled rabbit anti-mouse immunoglobulin (Ig) produced by one of the authors (M. Loken) has been described previously (16).

*Immunization Procedure.* BDF<sub>1</sub> mice were immunized five times, with each injection consisting of  $\sim 2 \times 10^7$  cloned L3 CTL cells. The first injection was given intraperitoneally in complete Freund's adjuvant followed at 3-wk intervals by intraperitoneal injections in incomplete Freund's adjuvant. Inhibitory effects on cytolysis of P-815 (*H-2<sup>d</sup>*) target cells by L3 cloned CTL could be detected in the serum of animals after the fourth injection. 3 d before fusion, a final intravenous injection of L3 cells was given.

*Fusion.* Spleen cells from immune BDF<sub>1</sub> mice were fused with SP2/0-Ag14 (SP2/0) hybrid cells using the polyethylene glycol fusion procedure described by McKearn et al. (17).

*Assays for Inhibition of Cytolysis.* Microinhibition assays for the screening and analysis of hybridoma supernatants were performed using the method of Dialynas et al. (11). Briefly, the effector cells were incubated with antibody for 30 min at 4°C, followed by the addition of <sup>51</sup>Cr-labeled target cells and incubation at 37°C for periods ranging from 30 to 90 min to induce the lytic injury. EDTA was then added to prevent further lysis, and cells were incubated for an additional period of 2–3 h to permit maximum release of <sup>51</sup>Cr. The effector/target cell ratio and incubation times were predetermined for each cloned CTL to provide the maximum sensitivity for detection of inhibition of cytolysis.

Inhibition of cytolysis of several cloned CTL and of primary MLC cells was determined over a range of effector/target cell ratios using a short-term <sup>51</sup>Cr-release assay described previously (10). This procedure was modified to test for inhibition of cytolysis under lectin-facilitated conditions by increasing the period of incubation before the addition of EDTA to provide for optimal specific <sup>51</sup>Cr release from the targets.

*Fluorescence Staining and Flow Cytometric Analysis.* Indirect immunofluorescence was used in all instances. Cloned CTL, normal tissue cells, or 5 d MLC cells were centrifuged over a Ficoll-Hypaque gradient (18), washed thoroughly in medium and distributed for fluorescence staining. A two-stage technique was used with staining at room temperature in the presence of

0.2% sodium azide. The first-step antibody was incubated under saturating conditions with  $10^6$  cells for 15 min in 25  $\mu$ l total volume. Cells were washed by the addition of 75  $\mu$ l Dulbecco's phosphate-buffered saline (DPBS) containing 5% agammaglobulinemic horse serum (AGH) over a cushion of 25  $\mu$ l AGH, and centrifuged at 600  $g$  for 15 min. The second step reagent was fluorescein-coupled rabbit anti-mouse Ig that was added under saturating conditions in a final volume of 25  $\mu$ l, and the cells were incubated and washed as described above. The stained cells were resuspended to a volume of 500  $\mu$ l in DPBS/5% AGH in the presence of propidium iodide (1.5  $\mu$ g/ml) (19).

Cells were analyzed using a fluorescence-activated cell sorter (FACS IV, B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, CA) equipped with a four decade logarithmic amplifier (Nozaki Associates, Inc., Palo Alto, CA). In all experiments,  $10^4$  viable cells, as determined by exclusion of propidium iodide, were analyzed (19).

Results obtained with experimental and control cell populations are presented as fluorescence histograms, with the number of cells on the y axis and the log of fluorescence intensity in arbitrary units on the x axis.

*Trypsin Treatment of L3 Cells.* Cloned L3 cells were harvested 6–7 d after the previous transfer, washed once in medium, and centrifuged over a 4-ml Ficoll-Hypaque gradient (18). Cells recovered from the interface were washed three times in medium and once in serum-free DME. Cells were resuspended in Hanks' balanced salt solution (without  $Ca^{++}$  or  $Mg^{++}$ ) at a concentration of  $2 \times 10^7$  cells/ml. An equal volume of trypsin (Miles Laboratories Inc., Research Products Div., Elkhart, IN) at various concentrations was added to the cell suspension, and the mixture was incubated at 37°C for 30 min. Ice-cold DME (10 ml) with 20% FCS was added to stop the reaction. The cells were washed three times in cold medium and counted. Overnight incubation of trypsin-treated cells was performed by culturing approximately  $5 \times 10^6$  L3 cells in 1 ml of medium in a plastic culture plate (76-033-05; Linbro Chemical Co.) The incubation was continued for 18 h at 37°C in a humidified 5%  $CO_2$  atmosphere.

*Mixed Lymphocyte Cultures.* Primary 5-d MLC cells were prepared as described previously (14). C57BL/6 responding cells ( $25 \times 10^6$ ) and irradiated (1,400 rad) stimulating cells ( $25 \times 10^6$ ) from various strains were combined in a final volume of 20 ml in plastic culture flasks (3024; Falcon Labware, Oxnard, CA). Cultures were allowed to incubate in a humidified 37°C incubator with a 5%  $CO_2$  atmosphere for 5 d.

Secondary MLC cells were generated from 12–14 d primary MLC by restimulating  $3.5 \times 10^6$  primary MLC cells with  $25 \times 10^6$  irradiated stimulating spleen cells from the appropriate strain. Responding cells were harvested for cytofluorometric analysis at 96 h following restimulation. Alternatively, 48 h after restimulation MLC cells were cloned as described (3) by limit dilution culture.

## Results

*Fusion Results.* Sera from two mice which had been immunized with the L3 CTL clone cells (as described in Materials and Methods) were found to inhibit the lysis of the P-815 target cells by L3 but not by B18 cells. Spleen cells from each of these mice were fused in separate fusions with myeloma cells, and the resulting hybrid cells were screened for clone-specific inhibition of cytolysis. The efficiency for the first fusion (microwells with growing cells per total microwells) was ~85% (816/960). The efficiency for the second fusion was ~82% (1,180/1,440). Two microwells from the first fusion and one microwell from the second fusion produced supernatants that inhibited the cytolysis of P-815 target cells by L3 but not B18 cells. One established hybridoma (designated FP384) was obtained from a microwell derived from the first fusion. This hybridoma was recloned, and the supernatants of that recloned line (designated 384.5) were used in the studies described below. Monoclonal antibody 384.5 is IgG3 (data not shown).

*The Clone-specific Nature of the Inhibition of Cytolysis by Monoclonal Antibody 384.5.* To examine the specificity of 384.5 blocking activity, CTL clones having distinct allo- or

modified-self-reactivity were tested in antigen-mediated cytolytic assays in the presence or absence of antibody 384.5. Fig. 1 shows the characteristic inhibition profiles of 384.5 monoclonal antibody on the antigen-mediated lysis of P815 target cells by the CTL clones L3 and B18, in the microinhibition assay (9). The cytolytic activity of CTL clone L3 (specific for  $H-2L^d$ , unpublished data) was inhibited in the presence of 384.5 antibody over a range of antibody concentrations, but the cytolytic activity of another CTL clone B18 (specific for  $H-2K^d$ ) was unaffected over the same range of antibody concentrations. In contrast, a potent anti-Lyt-2 monoclonal antibody, 3.155, blocked both L3 and B18 antigen-mediated lysis in a manner consistent with previously reported inhibition studies (9). The effects of an irrelevant monoclonal antibody (anti-Thy-1.2), 83A, are also shown for comparison in Fig. 1. The selective inhibition of L3 lytic activity by antibody 384.5 was tested over a range of effector to target cell ratios by using several cloned cytolytic T cell lines, including B18, T18 (which has cytolytic specificity for TNP-modified self [ $H-2^b$ ]-antigen), and T38 (a CTL line which reacts against TNP-modified self [ $H-2^b$ ]- or TNP-modified alloantigen [ $H-2D^d$ ]) (12). The results shown in Table I indicate that the monoclonal antibody 384.5 inhibited cytolytic activity of the CTL clone L3 over a range of effector/target cell ratios. Antibody 384.5 failed to block TNP-specific  $H-2D^d$  region-restricted lysis of TNP-modified P815 target cells by the CTL clone T38 or the lysis of TNP-modified EL-4 target cells by the CTL clone T18 (Table I). In contrast, the nonpolymorphic anti-Lyt-2 antibody, 3.155, strongly inhibited L3, T38, and T18 cytolytic activities against their appropriate targets. We have now tested >90 short-term cytolytic clones derived from alloreactive MLC that were not inhibited by the antibody 384.5 (data not shown).

Cytolytic activity of 5-d primary MLC cells is inhibited by treatment in the absence of complement with anti-Lyt-2 sera (20, 21), monoclonal antibodies (10), or with anti-

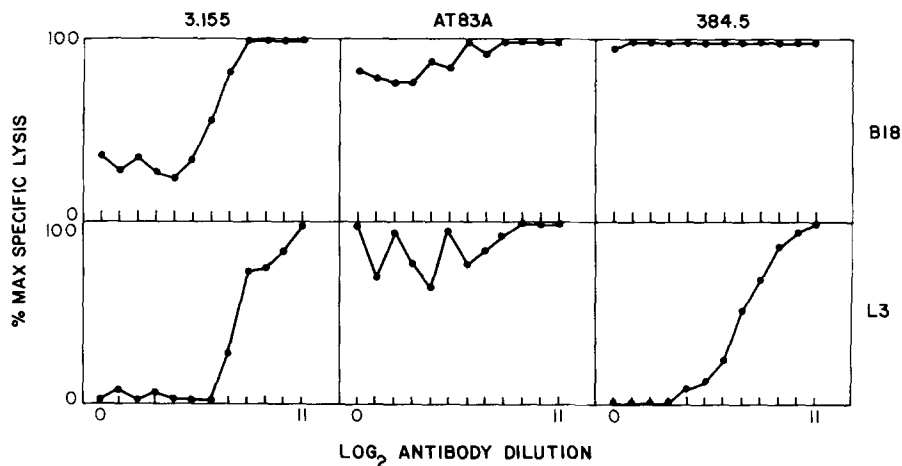


FIG. 1. Effects of monoclonal antibody 384.5 on lysis of P-815 target cells by CTL clones L3 and B18. A fixed number of L3 ( $5 \times 10^3$ ) or B18 ( $1 \times 10^4$ ) cells were preincubated for 30 min at  $4^\circ\text{C}$  with various dilutions of 3.155 (anti-Lyt-2), 83A (anti-Thy-1.2), or 384.5 supernatant fluids and tested for cytotoxicity against  $2.5 \times 10^3$   $^{51}\text{Cr}$ -labeled P-815 target cells. After the addition of the target cells, incubation at  $37^\circ\text{C}$  was continued for 90 min (B18) or 60 min (L3) before the addition of EDTA to inhibit further lytic activity. Incubations were continued at  $37^\circ\text{C}$  for a total of 180 min (B18) or 120 min (L3) before harvesting. Results are expressed as the percent lysis relative to that in the absence of antibody (9).

TABLE I  
*Effects of Monoclonal Antibody 384.5 on Lysis by CTL Clones L3, T18, and T38*

Target cell	Effector cell	E/T ratio	Monoclonal antibody added			
			Medium	3.155 (Anti-Lyt-2)	384.5	15E (Anti-Thy-1.2)
			Percent specific lysis*			
P-815-TNP	L3	20	62	6	5	54
		7	56	3	4	44
		3	37	3	3	43
	T38	20	77	5	86	71
		7	66	1	79	64
		3	47	6	66	53
C57BL/10-TNP	T18	20	76	2	74	71
		7	76	1	71	72
		3	62	0	68	66

\* Cloned CTL effector cells were preincubated with undiluted hybridoma culture supernatants 3.155 (anti-Lyt-2), 15E (anti-Thy 1.2), 384.5, or 20% AGH medium controls for 30 min. The appropriate target cells ( $5 \times 10^3$ ) were added and the mixture was incubated for 90 min before the addition of EDTA to inhibit further lytic activity. Incubations were continued for an additional 90 min. The results are expressed as the percent specific lysis at the indicated effector/target cell ratio.

170/100 (LFA-1) monoclonal antibodies (9, 22). To determine whether antibody 384.5 inhibited cytolysis by primary MLC cells, 5-d unidirectional C57BL/6 MLC cells responding to DBA/2, B10.D2, B10.A(5R), or CBA stimulating cells were tested in a short-term  $^{51}\text{Cr}$ -release cytotoxicity assay in the presence of monoclonal antibodies 384.5, 2.43 (anti-Lyt-2.2), 83A (anti-Thy 1.2), or media controls. The data shown in Table II indicate that antibody 384.5 did not strongly inhibit the cytolytic activity of MLC cells under conditions suitable for inhibiting cytolysis of L3 cells by other monoclonal antibodies. The effects of antibody 384.5 on the cytolytic activity of MLC cells stimulated twice in vitro are also shown in Table II. The results indicate that the cytolytic activity of MLC cells reactive against the  $H-2^d$  haplotype or against determinants controlled by the right half of the  $H-2^d$  haplotype [C57BL/6 anti-B10.A(5R)] were not strongly inhibited by antibody 384.5. However, cytolytic activity was significantly reduced when MLC cells were assayed in the presence of anti-Lyt-2.2 antibody 2.43.

Antigen-specific cytolytic T lymphocytes can be induced in the presence of the lectins, concanavalin A (Con A) or phytohemagglutinin (PHA), to lyse inappropriate target cells (23) by a mechanism similar to antigen-mediated cytolysis (24). CTL clones L3 and B18 were tested in lectin-facilitated  $^{51}\text{Cr}$ -release assays for their ability to lyse either syngeneic target cells, EL-4, or irrelevant allogeneic target cells, AKR-A, in the presence of antibody 384.5, an anti-Lyt-2.2 antibody (2.43), or medium. The data shown in Table III indicate that antibody 384.5 strongly inhibited Con A-facilitated cytolysis of both EL-4 and AKR-A target cells by the CTL clone L3. Interestingly, antibody 384.5 did not strongly inhibit PHA-facilitated cytolysis of AKR-A target cells by the L3 effector cells. We have also tested the specificity of inhibition of lectin-facilitated lysis by antibody 384.5. The data presented in Table III show that antibody 384.5 did not inhibit Con A-facilitated cytolysis of EL-4 or

TABLE II  
*Effects of Antibody 384.5 on the Cytolytic Activity of Bulk MLC Cells\**

Target cell	Effector cell	E/T ratio	Percent specific lysis				
			20% AGH (Medium)	83A (anti-Thy-1.2)	2.43 (anti-Lyt-2.2)	384.5	
P-815	L3	10	73	69	1	14	
		3	64	57	0	11	
Primary MLC P-815	B6 $\alpha$ -DBA/2	30	68	69	27	68	
		10	49	43	15	49	
		3	28	26	11	24	
	B6 $\alpha$ -B10.D2	30	71	74	25	65	
		10	61	61	14	57	
		3	45	35	10	36	
	B6 $\alpha$ -B10.A(5R)	30	65	68	20	54	
		10	52	58	17	44	
		3	36	38	5	30	
	AKR-A	B6 $\alpha$ -CBA	30	22	28	0	39
			10	20	17	0	30
			3	12	14	0	14
Secondary MLC P-815	B6 $\alpha$ -DBA/2	30	20	25	2	23	
		10	16	18	3	14	
		3	12	7	0	8	
	B6 $\alpha$ -B10.D2	30	24	26	2	27	
		10	17	20	1	20	
		3	10	8	1	9	
	B6 $\alpha$ -B10.A(5R)	30	23	37	3	29	
		10	25	20	4	25	
		3	19	15	3	13	

\* Effector cells obtained from the indicated MLC were preincubated with 20% AGH control or (undiluted) supernatants from hybridomas 83A (anti-Thy-1.2), 2.43 (anti-Lyt-2.2), or 384.5 for 30 min. The appropriate target cells were added to the culture and the lytic reaction was allowed to proceed for 45 min. Lysis was stopped after 45 min by the addition of EDTA and the incubation was continued for an additional 2 h.

AKR-A target cells by B18 effector cells. Furthermore, the lectin PHA did not efficiently induce the B18 cells to lyse AKR-A target cells.

*Specificity of Binding of Monoclonal Antibody 384.5.* We have investigated the expression of the cell surface determinant detected by monoclonal antibody 384.5 on cloned T lymphocytes, 5-d alloreactive MLC cells, and normal murine tissues by indirect immunofluorescence using flow cytometric analysis. Fig. 2A, shows the typical immunofluorescence profile observed with antibody 384.5 on L3 cloned CTL cells. The relative fluorescence intensity observed with antibody 384.5 was two and a half times greater than when L3 was reacted with an inappropriate third-party antibody (data not shown) or with the fluorescein-labeled second antibody alone. When the CTL clone B18 was tested under identical conditions the fluorescence histogram for

TABLE III  
*Effects of Monoclonal Antibody 384.5 on Lectin-facilitated Cytolysis by Cloned CTL L3 and B18*

Target cell	Lectin	Effector cell	E/T ratio	Monoclonal antibody added		
				Medium	2.43 (anti-Lyt-2.2)	384.5
				Percent specific lysis*		
EL-4	Con A	L3	20	45	50	10
		B18	20	47	40	43
AKR-A	Con A	L3	30	35	36	1
		B18	30	26	23	31
	PHA	L3	30	39	26	27
		B18	30	0	0	0

\* L3 and B18 cells were preincubated with undiluted hybridoma supernatant 2.43 (anti-Lyt-2.2), 384.5, or with 20% AGH medium controls for 30 min at 4°C before the addition of  $2.5 \times 10^3$   $^{51}\text{Cr}$ -labeled target cells. Incubations were continued at 37°C for 90 min in the presence of 20  $\mu\text{g}/\text{ml}$  Con A or 2  $\mu\text{g}/\text{ml}$  PHA before the addition of EDTA. Incubations were continued for a total of 180 min. The results are expressed as the percent specific lysis at the indicated effector/target cell ratios.

antibody 384.5 was found to superimpose that of the second antibody (Fig. 2B). These findings indicate that the antigenic determinant detected by antibody 384.5 is present on L3 cells but not on B18 cells. We have found no evidence of specific binding of antibody 384.5 to 11 other cytolytic or amplifier T cell clones or to the P-815 target cells (data not shown).

Normal tissues, including preparations of spleen, lymph node, thymus, and bone marrow cells from C57BL/6 and BDF<sub>1</sub> mice were tested for expression of the 384.5 determinant. The results shown in Fig. 2C indicate that antibody 384.5 did not stain a detectable portion of BDF<sub>1</sub> spleen cells. Similar results were obtained with each of the normal cell preparations of both C57BL/6 and BDF<sub>1</sub> origin.

*Evidence for the Clonal Distribution of Cells Bearing the Determinant Detected by Antibody 384.5.* If the antigenic determinant recognized by antibody 384.5 represents an idiotypic determinant on L3 cells, and if that idio type were relatively common in the response of C57BL/6 mice to the *H-2<sup>d</sup>* alloantigens, it might be possible to identify cells bearing this determinant in bulk MLC populations by using flow cytofluorometric analysis. Antibody 384.5 did not stain a detectable portion of primary MLC cells including 5-d unidirectional C57BL/6 spleen cells responding to DBA/2, B10.D2, B10.A(5R), or CBA/J stimulating cells (data not shown). Fig. 3 shows the results of a representative experiment in which antibody 384.5 was reacted with C57BL/6 spleen cells that had been stimulated twice in MLC. The fluorescence profile obtained with C57BL/6 anti-CBA/J MLC cells when examined with antibody 384.5 superimposed the negative control (9). When C57BL/6 anti-B10.A(5R) MLC cells were examined (a combination that genetically enriched for MLC cells reactive with the *H-2D<sup>d</sup>* region gene products), a definite, though small population of cells reacted with the antibody 384.5. Similar results were obtained with three separate preparations of C57BL/6 anti-B10.A(5R) MLC cells.

*Evidence for the Endogenous Synthesis of the Determinant Detected by Antibody 384.5.* As a preliminary step in determining the nature of the molecule bearing the determinant detected by antibody 384.5, the susceptibility of that determinant to proteolytic digestion by trypsin was investigated. The loss of expression of the determinant by L3

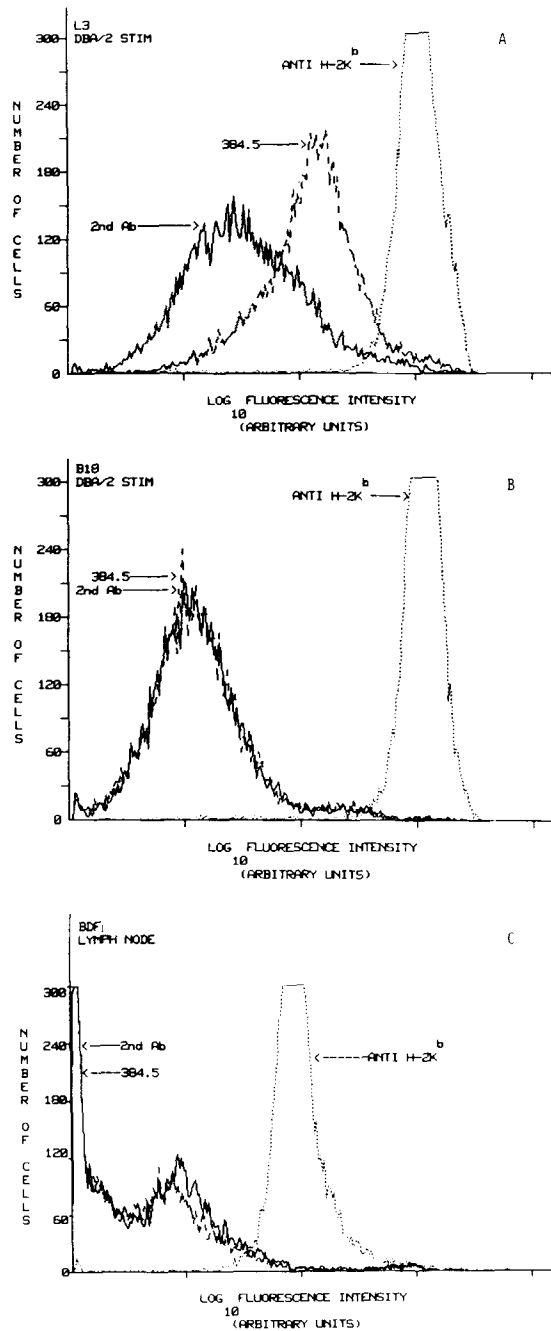


FIG. 2. Flow cytofluorometric analysis of the antigenic determinant recognized by antibody 384.5 on two cytolytic T lymphocyte clones and on normal BDF<sub>1</sub> lymph node cells. Cells were centrifuged over a discontinuous Ficoll-Hypaque gradient, washed extensively, and distributed for fluorescence staining. A two-stage technique was used with staining at room temperature in the presence of 0.2% sodium azide. The first stage antibody, 384.5, or control monoclonal reagent as indicated was incubated under saturating conditions with 10<sup>6</sup> cells for 15 min. The second-stage antibody was a fluorescein-conjugated rabbit anti-mouse Ig in all experiments. Cells were analyzed by flow cytofluorometry using a modified FACS IV as described in the text. In all experiments, 10<sup>4</sup> live cells as determined by exclusion of propidium iodide were analyzed. Results are expressed in histogram form with the ordinate representing cell number, and the abscissa representing the log of fluorescence intensity in arbitrary units where one decade is represented by each gradation along the x axis.



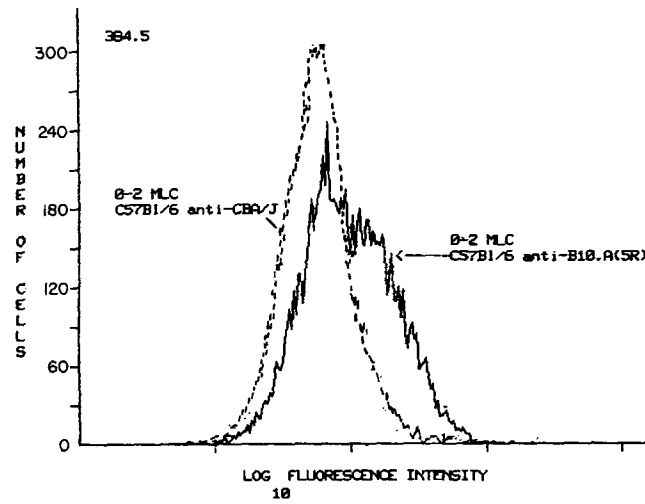


FIG. 3. Flow cytometric analysis of the antigenic determinant recognized by antibody 384.5 on secondary MLC cells. C57BL/6 anti-CBA/J and C57BL/6 anti-B10.A(5R) MLC cells were stimulated twice in vitro and then examined for expression of the antigenic determinant detected by antibody 384.5. The MLC combination C57BL/6 anti-B10.A(5R) genetically enriches for CTL specific for the *H-2D<sup>d</sup>* region. Preparation and staining methods are described in the legend for Fig. 2. The second-step reagent was a fluorescein-conjugated rabbit anti-mouse Ig. (9).

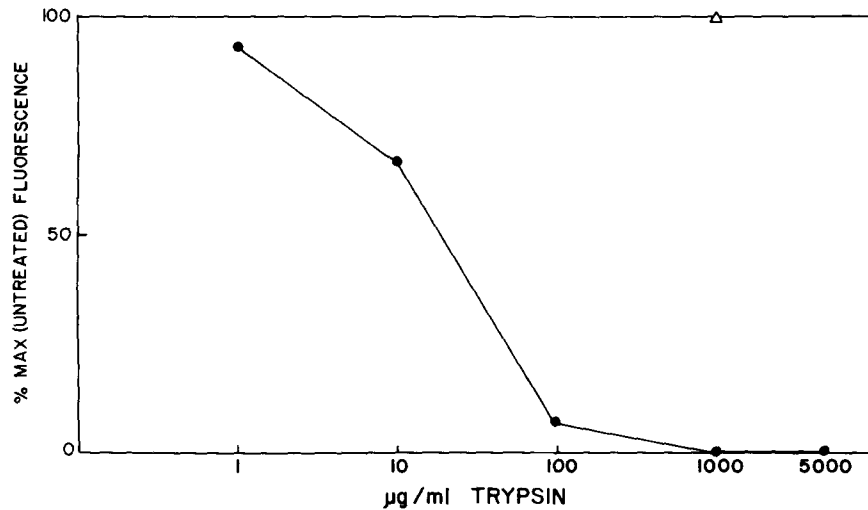


FIG. 4. Effect of trypsin treatment of L3 cells on the relative expression of the determinant detected by antibody 384.5. Cloned L3 cells were treated with the indicated concentrations of trypsin as described in Materials and Methods. Cytofluorometric analysis (as described in Fig. 2) was used to determine the ability of antibody 384.5 to bind to freshly trypsinized L3 cells (●) or to L3 cells treated with trypsin (1000 µg/ml) and then incubated overnight in medium (Δ). The results are expressed as the percent maximum (untreated) L3 cell fluorescence with antibody 384.5.

as a result of trypsin treatment was analyzed using indirect immunofluorescence and flow cytometric analysis. The results presented in Fig. 4 show that the relative fluorescence intensity of the L3 cells stained with antibody 384.5 (expressed as a percentage of the fluorescence intensity of untrypsinized L3 cells stained with antibody

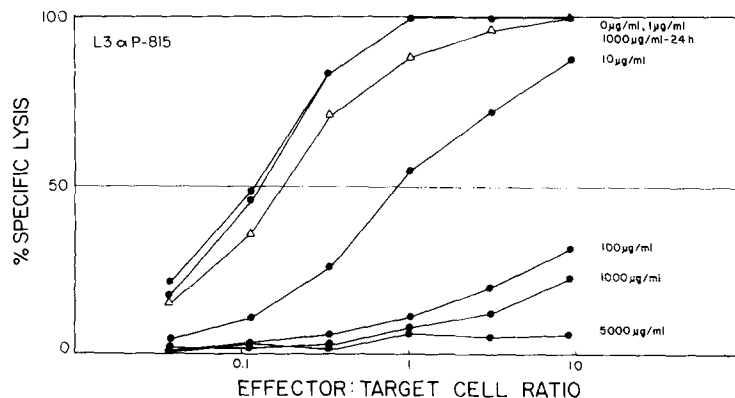


FIG. 5. Effect of trypsin treatment of L3 cells on their cytolysis of P-815 target cells. L3 cells were treated with the indicated concentrations of trypsin as described in Materials and Methods. The relative cytolytic efficiency of the freshly trypsinized L3 cells (●) or of L3 cells treated with trypsin (1,000  $\mu\text{g}/\text{ml}$ ) and then incubated overnight in medium ( $\Delta$ ) was determined in a 3.5 h antigen-mediated cytolytic assay using  $5 \times 10^3$  P-815 target cells. The results are expressed as the percent specific lysis at various effector/target cell ratios.

384.5) could be reduced to the level of staining with second antibody alone by treatment for 30 min with trypsin concentrations  $\geq 1,000 \mu\text{g}/\text{ml}$ .

To determine if L3 cells were capable of reexpressing the determinant detected by antibody 384.5 after enzymatic treatment, L3 cells treated with trypsin at a concentration of 1,000  $\mu\text{g}/\text{ml}$  were incubated overnight in medium without filler cells at 37°C. The results, shown in Fig. 4, indicate that after overnight incubation the L3 cells stained with a fluorescence intensity equivalent to that seen with untrypsinized control L3 cells. Similar results were observed in three separate experiments.

The effect of trypsin proteolysis on L3 cell cytolytic activity was also determined with the same cell preparations used for the analysis of enzyme susceptibility of the 384.5 determinant. The effect of trypsin treatment on the capacity of the cloned L3 cells to lyse P-815 target cells is shown in Fig. 5. The results indicate that the cytolytic efficiency of L3 cells decreased as a function of the increasing trypsin concentration. L3 cells treated with 1,000  $\mu\text{g}/\text{ml}$  of trypsin and incubated overnight in medium recovered their cytolytic potential.

### Discussion

Our approach in this study was designed to obtain monoclonal antibodies capable of inhibiting cytolytic activity of cloned CTL in a clone-specific manner. We have used the CTL clone L3 to immunize  $F_1$  animals by using a procedure similar to that reported by Binz and Wigzell (6) to produce "antiidiotypic" antisera. The choice of an  $F_1$  hybrid mouse, a cross between the strain of origin of the effector cell and the strain of origin of the target cell, should favor the development of clone-specific antibodies. This situation minimizes the opportunity for production of antibodies reactive with Lyt-2, 170/100, MHC, and other antigens shared by the CTL clone and the mouse being immunized. It also lessens the chance for production of antibodies having specificity for target cell antigens which might be adsorbed to the cloned CTL used in the immunization (25).

The antigen-mediated cytolytic process of CTL has been resolved into at least three

steps (26): recognition-adhesion, the delivery of the lethal hit, and target cell destruction. Recognition-adhesion may represent two separate steps, but they have not been functionally resolved. Recognition of antigen is an essential element of the antigen-mediated cytolytic process, and antibodies directed against the recognition structures on the CTL cell surface might be expected to interfere with that process. In the present study, we used a microinhibition screening procedure that was developed in this laboratory to provide a sensitive means of detecting monoclonal antibodies reactive with cell surface structures associated with the cytolytic process (11). By combining the F<sub>1</sub> immunization procedure and the functional screening assay, we identified the monoclonal antibody 384.5 that inhibits the cytolysis by the CTL clone L3 but does not inhibit cytolysis by other CTL clones. We have now tested the specificity of inhibition of cytolysis by antibody 384.5 with >90 short-term cytolytic clones derived from C57BL/6 anti-B10.A(5R) MLC; none of these clones were inhibited by the antibody 384.5.

The specificity of inhibition of cytolysis by antibody 384.5 correlated well with the specificity of antibody binding as determined by flow cytofluorometry. The determinant detected by antibody 384.5 is expressed by the L3 CTL clone, but not by 12 other cytolytic or amplifier T lymphocyte long-term clones, nor was it expressed on a detectable portion of cells derived from normal tissues or alloreactive primary MLC of C57BL/6 origin.

The clone-specific nature of the reactivity of antibody 384.5 distinguishes this antibody from other monoclonal antibodies derived in our laboratory such as anti-Lyt-2, anti-170/100, or anti-H-2 antibodies that inhibit lytic activity of CTL clones nonspecifically (9). Furthermore, our cytofluorometry data suggest that the determinant detected by antibody 384.5 is expressed at a cell surface density much lower than that of the Lyt-2 molecular complex. Although the determinant detected by antibody 384.5 is distinct from those detected by the other antibodies that inhibit cytolysis, the nature of the molecule recognized by antibody 384.5 remains to be determined. It has not been possible thus far to obtain immunoprecipitates with antibody 384.5. This difficulty may relate to the low level of expression of the determinant with which this antibody reacts.

Our results indicate that the determinant detected by antibody 384.5 is removed by proteolytic digestion. Recent studies in this laboratory have shown that antigenic structures, including molecules bearing class I and class II determinants, can be adsorbed to the surface of cloned cytolytic and amplifier T lymphocytes (25). The determinant detected by antibody 384.5 does not appear to be acquired in this way. The capacity of enzymatically treated L3 cells to reexpress the 384.5 determinant after overnight incubation indicates that the cell surface molecule bearing this determinant is endogenously synthesized by the L3 cells.

There are several potential mechanisms by which the antibody 384.5 may inhibit L3 cell cytolytic activity. The results of the binding studies suggest that the antibody inhibits cytolysis by binding specifically to the L3 effector cell and not to the target cell. Furthermore, these studies suggest that inhibition is probably not due to nonspecific "blanketing" effects from high antigen density, because the cytofluorometry studies indicate a relatively low antigen density on the surface of L3 cells. Antibody 384.5 is not directly cytotoxic to L3 cells, nor does it appear to induce effector cell autolysis under the conditions of the blocking assay as determined by

<sup>51</sup>Cr-release assays or trypan blue exclusion assays (unpublished data). Antibody 384.5 does not appear to inhibit cytolysis by binding directly to the molecule that delivers the lethal hit because this antibody specifically inhibits L3 cytolytic activity but not the cytolytic activity of other CTL clones. Furthermore, antibody 384.5 did not strongly inhibit PHA-facilitated cytolysis of AKR-A target cells by L3 effector cells. The results of preliminary studies on the effects of antibody 384.5 on antigen-specific conjugate formation between L3 cells and P-815 target cells suggest that this antibody inhibits L3 cell lysis of P-815 target cells by interfering with the recognition-adhesion step of the cytolytic process.

If the molecule bearing the determinant recognized by antibody 384.5 is involved in antigen recognition, then enzymatic removal or alteration of that molecule might be expected to result in the loss of antigen-mediated cytolytic activity. The simultaneous loss by L3 cells of antigen-specific cytolytic activity and the expression of the antigenic determinant detected by antibody 384.5 suggest that both properties have similar sensitivities to trypsin treatment. Results obtained in experiments that compare the susceptibility of L3 cell antigen-mediated cytolysis and lectin (Con A)-facilitated cytolysis to trypsin treatment (unpublished data) suggest that the L3 CTL clone antigen-mediated and lectin-facilitated cytolytic activities also have similar sensitivities to trypsin treatment. Berke et al. (27) have suggested that the CTL antigen receptor may play a role in cell-to-cell interactions that occur in lectin-facilitated lysis of irrelevant target cells. If this is true, and if antibody 384.5 reacts with a determinant on the T cell receptor, the ability of antibody 384.5 to inhibit both antigen-mediated and Con A-facilitated lysis is to be expected.

The monoclonal antibody 384.5 represents a reasonable candidate for an antibody directed against a determinant on the CTL receptor. This antibody binds specifically to and inhibits cytolysis by the CTL clone L3 but does not bind to or inhibit several other CTL clones. If antibody 384.5 recognizes an idiotypic determinant on the L3 cell antigen receptor, this idio type does not appear to be dominant among the C57BL/6 T cell clonotypes that respond to *H-2<sup>d</sup>* haplotype stimulation. Cells from primary 5-d MLC which react with antibody 384.5 have not been detected with certainty using flow cytometric analysis (data not shown). However, when the cell populations are derived from MLC that were genetically "enriched" for cells reactive with determinants controlled by the *H-2D<sup>d</sup>* region [C57BL/6 anti-B10.A(5R)] a small portion of these cells have been regularly observed to stain with antibody 384.5. It is possible that these cells represent a clonally expanded subpopulation that share an idiotypic determinant with the L3 CTL clone.

Antisera that appear to have antiidiotypic antibodies reactive with hapten-specific (7) or viral-specific (8) CTL have been produced by immunizing syngeneic mice with MLC cells that have cytolytic activity. Specificity for the appropriate CTL cells was suggested in these studies by the ability of these antisera to reduce cytolytic activity in the appropriate MLC cell preparations by treatment with antiserum and complement (7, 8). However, none of these antisera inhibited cytolytic activity in the absence of complement (8). Recently, antiidiotypic antisera have been produced by immunizing F<sub>1</sub> mice with cloned alloreactive noncytolytic T cells (28). Although these antisera stimulated clone-specific proliferation, they appear also to contain antibodies which bind to irrelevant clones as well as the specific clone (C. G. Fathman, personal communication).

The clone-specific nature of the reactivity exhibited by the monoclonal antibody 384.5 for a cytolytic T cell appears to be unique. This clone-specific reactivity of antibody 384.5 was demonstrated both in its capacity to inhibit L3 cytolysis and in its ability to bind to L3. The specific pattern suggests that this antibody may be reacting with the antigen-binding region of the antigen receptor on L3 CTL.

### Summary

Monoclonal antibody 384.5 specifically inhibited cytolysis of P-815 target cells by cloned L3 cytotoxic T lymphocyte (CTL) effector cells. The lytic activity of other cloned CTL that have other distinct specificities was not affected. Antibody 384.5 did not inhibit the cytolytic activity of bulk populations of C57BL/6 mixed lymphocyte culture (MLC) cells. Concanavalin A-facilitated cytolysis by T cell clone L3 but not T cell clone B18 was inhibited by antibody 384.5, whereas phytohemagglutinin-facilitated cytolysis by L3 cells was not strongly inhibited. Antibody 384.5 binds specifically to L3 cells but not to several other T lymphocytes clones, or to a detectable portion of populations of primary MLC cells, normal spleen, thymus, lymph node, or bone marrow cells. In contrast, C57BL/6 anti-B10.A(5R) secondary MLC cells (genetically enriched for reactivity against the *H-2D<sup>d</sup>* region gene products) contained a small population which reacted with the antibody 384.5. The determinant detected by antibody 384.5 was susceptible to trypsin treatment, and was reexpressed after overnight incubation. These results suggest that the monoclonal antibody 384.5 detects an endogenously synthesized clone-specific determinant associated with the cytolytic activity of the L3 CTL clone. These properties make antibody 384.5 an attractive candidate for an antibody that reacts with the antigen-recognition site of a cytolytic T cell antigen receptor.

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