Title

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Journal

The Journal of Immunology, 119(2)

ISSN

0022-1767

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Publication Date

1977-08-01

DOI

10.4049/jimmunol.119.2.374

THE JOURNAL OF IMMUNOLOGY

Vol. 119, No. 2, August 1977
Printed in U.S.A.

INHIBITION OF HUMAN LYMPHOCYTE-MEDIATED MITOGEN-INDUCED CYTOTOXICITY OF MURINE L-929 CELLS BY HETEROLOGOUS ANTI-HUMAN LYMPHOTOXIN ANTISERA *IN VITRO*[1]

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Heterologous anti-human lymphotoxin (LT) antisera have been employed to investigate the role of LT in mitogen-(Con-A, PHA) induced destruction of murine L-929 cells by human lymphocytes *in vitro*. These various antisera will effectively neutralize human LT molecules associated with the stable (70 to 90,000 dalton) α-LT class of cytotoxin (anti-α-LT), the more unstable (35 to 50,000 dalton) β-LT class of cytotoxins (anti-β-LT), and antisera which will neutralize all classes of these cytotoxins *in vitro*, anti-whole supernatant (anti-W.S.). These anti-LT sera will greatly inhibit lysis of L-929 cells by using mitogen-activated human effector lymphocytes *in vitro*. This blocking was shown to be mediated by whole serum, purified IgG, or IgG-Fab fragments, which had been extensively absorbed with bovine serum, human serum, mitogens, and normal human lymphocytes. Inhibition of lysis was not apparently due to interference with either lymphocyte-target cell contact or lymphocyte activation step(s). The blocking effects of these sera were also shown to occur during the lymphocyte-independent phase of the lytic reaction. These data support the concept that the lymphocyte deposits an LT-like effector molecule on the target-L cell surface during the lymphocyte-dependent phase, which mediates cell lysis at a later time during the lymphocyte-independent phase.

Our understanding of the cell and molecular mechanisms involved in target cell destruction mediated by immune or mitogen-activated effector lymphocytes *in vitro* is of central importance to our understanding of the mechanism involved in lymphocyte-mediated tissue destructive immune reactions *in vivo*. These latter *in vivo* reactions are typified by the tissue destruction observed during allograft rejection, tumor immunity, and certain autoimmune disease states.

The biochemical mechanisms involved when "killer" lymphocytes effect direct target cell lysis *in vitro* remain unclear. It is a complex situation involving different classes of effector cells (1, 2) and potentially different types of lytic mechanisms (3, 4). However, several aspects of direct lymphocyte-mediated cytotoxicity have been established: 1) physical contact between the aggressor lymphocyte and target cell must occur (*lymphocyte dependent*, temperature-independent phase (5)); and 2) target cell lysis can occur subsequently in the absence of the effector lymphocyte during the *lymphocyte-independent*, but temperature-dependent phase (6–8).

It has been proposed by several investigators that target cell lysis may involve the secretion of nonspecific soluble effector molecule(s), termed lymphotoxin(s) (LT)[2] (9–11). Indeed, it has been demonstrated in a number of laboratories that incubation of immune lymphoid cells with specific (soluble) antigen, nonimmune lymphocytes with mitogens, or mixed lymphocyte reactions *in vitro* results in the elaboration of such a cytotoxic agent (12–16). It has been suggested by Kramer and Granger (17) that destruction of mouse L cells *in vitro* by mitogen-activated human lymphocytes or by supernatants obtained from activated human lymphocytes occurs via a common agent(s) since 1) both reactions are very temperature sensitive with both being blocked at the nonpermissive cytolytic temperature of 34°C; and 2) trypsinization of target cells after contact with activated lymphocytes can protect a significant amount of cytotoxicity.

Human lymphotoxins are a complex family of biologic macromolecules. Recent physical studies have shown that these molecules can be fractionated into at least two major m.w. classes, termed α-LT and β-LT (18, 19). Additional studies have shown that each m.w. class in turn contains multiple subclasses (3–4 in the α-LT(20) and 2–3 in the β-LT (21)), which appear to be immunologically distinct (manuscript in preparation). Because of this extensive heterogeneity and the difficulty in developing a full spectrum of monospecific sera against each purified subclass of LT, this study employed more broadly reactive sera which ensured complete neutralization of all members within a given class of LT molecules. Therefore, we developed antisera directed against narrow m.w. Sephadex fractions containing *all* members of the α-LT class (anti-α-LT), *all* the members of the β-LT class (anti-β-LT), or antisera which neutralize all classes of human lymphotoxins *in vitro* (anti-W.S.) (22). In these studies, we have employed these anti-LT sera to investigate the role of various classes of these cytotoxins in the lysis of murine L-929 cells by mitogen-stimulated human lymphocytes *in vitro*.

Two major theories presently exist as to the nature of the mechanism of direct cytolysis: 1) the lymphocyte confers an irreversible lesion to the target cell in the absence of lytic effector molecules (23, 24); 2) the lymphocyte binds to and deposits cytotoxic molecules on the target cell surface, which can mediate destruction at a later time during the lymphocyte independent phase (17). These results suggest that LT molecules do play a major role in the mechanism of target cell lysis *in vitro*. In addition, it appears that once deposited on the

Received for publication November 22, 1976.
Accepted for publication April 11, 1977.
[1] This work was supported by Grant AI-09460 from the Institute of Allergy and Infectious Diseases, National Institutes of Health, Grant IM-32 from the American Cancer Society, and Grant 1883 from the Rheumatic Diseases Research Foundation.

[2] Abbreviations used in this paper: LT, lymphotoxin; MICC, mitogen-induced cellular cytotoxicity; NRS, normal rabbit serum; MLC, mixed lymphocyte culture reactions; anti-LT, anti-lymphotoxin; anti-α-LT, Sephadex G-150 fraction free of β-LT; anti-β-LT, Sephadex G-150 fraction free of α-LT; anti-W.S., whole unfractionated supernatants prepared in serum-free media.

surface of the target L cell after contact with the effector lymphocyte, LT can mediate the lytic events in the absence of the lymphocyte, during the independent phase.

MATERIALS AND METHODS

Target cells and culture media. Stock cultures of a sensitive strain of mouse L-929 fibroblasts (α-L-929), were used as target cells (25). These cells were maintained in 32-oz prescription bottles in 95% air, 5% CO_2, and passed biweekly. Culture media consisted of minimal essential media with Hanks' salts, supplemented with 3% heat-inactivated (56°C, 60 min) fetal calf serum (Microbiological Associates, Bethesda, Md.), 0.2 μg/ml glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) (MEMS).

Lymphocyte cultures. Suspensions of human small lymphocytes from tonsils or adenoids were obtained within 6 to 8 hr of surgical removal from normal children, as previously described (26). Lymphocyte suspensions were adjusted 4×10^6 viable cells/ml in 32-oz prescription bottles, with MEMS, and incubated for 3 to 5 days in 95% air, 5% CO_2 at 37°C. Activation of the lymphocytes was effected by the addition of phytohemagglutinin-P (PHA-P, Difco Laboratories, Detroit, Mich.) at 20 μg/ml or concanavalin A (Con A, Sigma, St. Louis, Mo.) at 5 μg/ml. When employed as effector cells, PHA-activated lymphocytes were pipetted vigorously to break up clumps and washed two times with MEMS before use. To those cultures activated with Con A, α-methyl-D-mannoside (α-MAM) was added to 5 mM, the cells were vigorously pipetted, and then washed two times (using 40 ml each) in MEMS plus 5 mM α-MAM to remove all residual cell-bound Con A.

Direct lymphocyte-mediated cytotoxicity. Target L cells (10^5 cells in 1.0 ml MEMS containing 0.5 μg/ml mitomycin C) were established 24 hr before use, in 16- x 125-mm screw-capped tubes. The media were discarded, the monolayers were washed with PBS, and to each tube, 1 ml of a cell suspension containing a predetermined number of lymphocytes was added. Ratios typically employed were 0.05, 0.5, 2, and 10 lymphocytes to 1 target cell, as indicated in the text. Con A was then added to each tube at 5 μg/ml or Con A plus 5 mM α-MAM was added as controls. The tubes were then incubated at 37°C for 24 hr, after which they were vigorously shaken and the dead cells, which had detached from the monolayers, were poured off in the supernatant. The remaining viable adherent L cells were removed and counted as previously described (27). Mitogen-coated target cells were also employed in these studies. Target L cells were coated with PHA-P at 100 μg/ml or control MEMS for 30 to 60 min at 37°C and washed twice with 2.0 ml PBS. Various numbers of lymphocytes in 1.0 ml MEMS were then added as described above.

Lymphocyte-dependent: independent phase of MICC. Effector lymphocytes were harvested, washed twice in 20 volumes of MEMS, resuspended in fresh MEMS, and placed on target L cell monolayers at various lymphocyte to target cell ratios in the presence of: 1) fresh Con A (5 μg/ml); 2) Con A plus 5 mM α-MAM; or 3) PHA-P-coated target cell monolayers. After various periods of time at 37°C, as indicated in the text, the lymphocytes were removed by vigorous shaking and the monolayers were washed twice in 2.0 ml PBS. We found this procedure routinely removed > 96% of the bound lymphocytes without affecting the number of attached L cells. Antisera or normal rabbit serum (NRS) (100 to 200 μl) were then added, and the cultures were allowed to incubate at 37°C for an additional 24 hr.

Preparation and use of anti-LT antisera. The methods for obtaining and testing specific anti-LT antisera have been previously reported (22). Preparations of α-LT or β-LT used as immunogens were obtained from supernatants of 5-day PHA-P-activated human tonsil and adenoid lymphocytes cultured in RPMI 1640 with 20 μg/ml serum substitute (28). Anti-LT antisera were generated in New Zealand White rabbits after repeated injections of one of the following preparations of LT suspended in complete Freunds adjuvant: a) a narrow m.w. fraction of material (70 to 90,000 daltons) from Sephadex G-150 (2.5- x 100-cm column) that, after rechromatography on a second indentical Sephadex G-150 column, contains *all* members of the α-LT class of cytotoxins and is completely free of contaminating β-LT activity (anti-α-LT); b) a narrow m.w. fraction of material (35 to 50,000 daltons) from Sephadex G-150 that, after rechromatography on a second Sephadex G-150 column, contains *all* members of the β-LT cytotoxins, and is free of contaminating α-LT (21) (anti-β-LT); and c) whole unfractionated supernatants prepared in serum-free media (anti-W.S.). These latter antisera will completely neutralize cytotoxic activity associated with all known human LT molecules *in vitro*.

The above antisera or NRS were collected, heat inactivated (56°C, 45 min) and filter sterilized before use. In several cases, preimmune rabbit serum was obtained and employed in place of NRS, yielding identical results. For these experiments, antisera were used at a final concentration of 10 to 20%, as indicated in the text. Percentage of neutralization was determined with the following formula:

$$\% \text{ Neutralization} = \frac{(\text{Cell No.} + \text{antiserum}) - (\text{cell No.} + \text{NRS})}{(\text{Cell No.} + \text{normal media}) - (\text{cell No.} + \text{NRS})} \times 100.$$

Preparation of IgG and Fab fragments. Test and control sera were slowly brought to 40% concentration of ammonium sulfate, pH 7.2, at 4°C. The resulting precipitate was solubilized in 0.15 M NaCl solution, then dialyzed against 0.15 M NaCl in 0.01 M phosphate, pH 7.2 (PBS). A suspension of mouse L cells in PBS was obtained by scraping confluent stock monolayers, and each solution received 10×10^6 L cells/4 mg of protein. After 12 hr at 4°C, the cells were removed by centrifugation and the supernatant was dialyzed against 0.025 M NaCl, 0.01 M Tris, pH 8.0, and chromatographed over a 3.4 x 20 cm DEAE-cellulose column. The fractions containing IgG were collected, pooled, concentrated by ultrafiltration, and dialyzed against PBS. Double diffusion and immunoelectrophoresis revealed the preparation contained only IgG. Each sample (100 mg) was then incubated with 1.4×10^9 human lymphocytes per milliliter for 12 hr at 4°C, after which the cells were removed by centrifugation. Five milligrams each of PHA-P, bovine serum and human serum were linked covalently per milliliter to Sepharose 4B by the method of March *et al.* (29). Each sample was then passed sequentially over individual 10-ml columns of Sepharose 4B linked to one of the above proteins. Double diffusion analysis against the above materials revealed no bands of precipitation after absorption. Monovalent Fab fragments were prepared by pepsin cleavage of IgG fractions, as described by Williams and Chase (30). These fragments were then reduced, alkylated, and dialyzed against PBS, distributed in small aliquots, and stored at −20°C.

Measurement of lymphocyte DNA synthesis. Human tonsil- or adenoid-derived lymphocytes were cultured in microplates (Falcon, Oxnard, Calif.) at 5×10^5 cells/well (0.1 ml) in MEMS (10% FCS). Activation of the cells was effected by the

addition of 0.1 ml of various concentrations of Con A, PHA-P, or control media to give final concentrations of those indicated in the text. Mixed lymphocyte culture reactions (MLC) were initiated by mixing lymphocytes obtained from two unrelated donors at 1:1 ratios. IgG fractions of various antisera, NRS, or control media were then added to each well (25 μl) and the microplates were incubated for 72 hr at 37°C in 95% air, 5% CO_2. DNA synthesis was then measured during a 6-hr pulse by using 1 μCi/well of ^{3}H thymidine (New England Nuclear, Boston, Mass.) and the cells in each well were processed in a MASH II unit. The dried filters were then added to 3 ml of an omnifluor/toluene scintillation cocktail and counted on a Beckman Model LS33 scintillation counter.

RESULTS

Inhibition of mitogen-induced cellular cytotoxicity (MICC) by anti-lymphotoxin antisera (anti-LT) with mitogen preactivated human effector lymphocytes in vitro. Experiments were initiated to investigate the capacity of anti-LT antisera to inhibit MICC of L-929 target cells *in vitro*. The first studies employed 3- to 5-day mitogen- (Con A or PHA-P) preactivated human lymphocytes as effector cells and two different agents to induce cytotoxicity during the MICC assay: 1) soluble Con A; and 2) PHA-coated target L cells. As can be seen in the data presented in Figure 1*A*, target cell lysis is observed when soluble Con A is employed as the inducing agent. Nearly 100% cytotoxicity is obtained at lymphocyte to target cell (Ly:TGC) ratios as low as 1:1. However, this effect is greatly reduced when a competitive inhibitor of the Con A-binding site (α-MAM) is employed. Figure 1*B* shows the same experiment in the presence of various concentrations and classes of anti-LT antisera. Antisera directed specifically at the 70 to 90,000 m.w. class of cytotoxins, α-LT (anti-α-LT) can inhibit a significant amount of cytotoxicity at a final dilution of 1/20 (50 μl), whereas 150 μl yields virtually complete target cell protection. Anti-W.S. is also effective at blocking MICC in this system. This inhibition is a dose-related phenomenon since high lymphocyte to target cell ratios override a constant amount of antisera, whereas increasing the amount of sera increased blocking. Inhibition of MICC in the presence of anti-LT antisera does not appear to be due to an inability of the lymphocytes to bind to the target cell, since microscopic observation verifies that good lymphocyte-target cell contact does occur. Table I shows the results of five different experiments in which Con A-activated human lymphocytes were employed as effector cells and soluble Con A was used as the inducing agent. In all experiments, good protection is observed when either anti-W.S. or anti-α-LT antisera is employed. In addition, these antisera will also neutralize growth inhibitory activities since experiment 5 used growing L cells as targets.

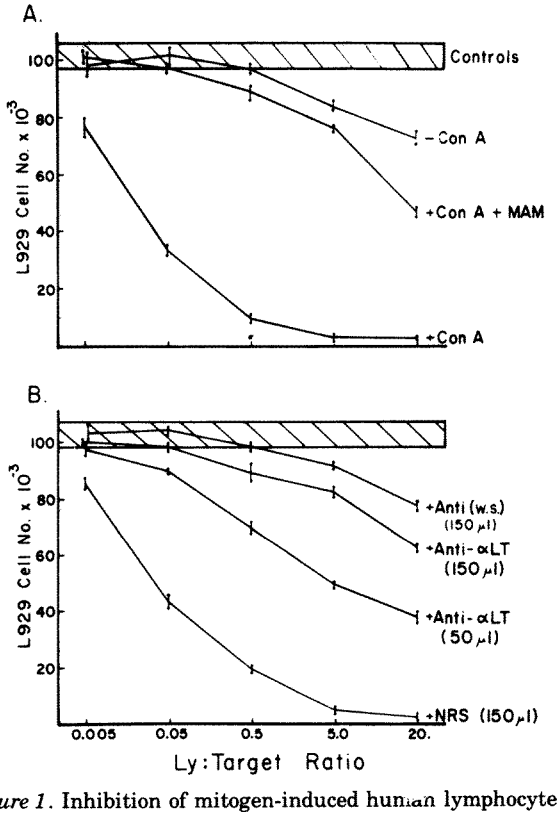

Figure 1. Inhibition of mitogen-induced human lymphocyte cytotoxicity (MICC) of L-929 TGC by various anti-LT antisera. *A*, Various numbers of 3 to 5 day Con A (5 μg/ml) preactivated human lymphocytes in 1 ml were placed on tube culture monolayers containing 10^5 mitomycin-C treated L-929 TGC in the presence (+ Con A) or absence (− Con A) of soluble Con A (5 μg/ml) or Con A plus a competitive binding inhibitor, α-MAM (5 mM) for 24 hr at 37°C. The lymphocytes were then physically removed by shaking, the monolayers washed, and the remaining adherent L cells enumerated on a Coulter counter as described in *Materials and Methods*. *B*, Same as in *A*, except that in addition to Con A, various amounts of anti-LT antisera were added as indicated. After 24 hr at 37°C, the remaining adherent L cells were enumerated on a Coulter counter.

Figure 2 shows the effects of these various antisera on MICC with PHA-coated L cells as targets. In this system, intimate contact between effector lymphocyte and target cell is required for lysis to occur. Figure 2*A* shows that when Con A-preactivated human lymphocytes are employed as effector cells, good blocking is obtained with both anti-W.S. and anti-α-LT antisera if 150 to 200 μl of antisera are employed. Identical results are obtained when PHA-preactivated lymphocytes are employed as effector cells in this system (Fig. 2*B*).

Inhibition of MICC by anti-LT antisera with unstimulated human lymphocytes as effector cells in vitro. The capacity of anti-LT antisera to block MICC of PHA-coated L cells by using

TABLE I

Effects of various anti-LT antisera on MICC of L cells by Con A preactivated human lymphocytes in vitro[a]

Expt.	Ly:TGC	% Viability		
		NRS	Anti-W.S.	Anti-α-LT
1[b]	5:1	6	92 (90)[c]	83 (80)
2	2:1	3	62 (60)	55 (52)
3	0.2:1	8	98 (96)	87 (78)
4	5:1	5	82 (80)	70 (66)
5[d]	10:1	48	91 (90)	75 (52)

[a] Human tonsil or adenoid lymphocytes were established in culture at 4 × 10^6/ml and activated with 5 μg/ml Con A as described in *Materials and Methods*. Three days later, the cells were collected, washed with 5 mM α-MAM to remove all Con A, the viability determined, using 0.1% eosin Y (routinely >80%), and various concentrations of lymphocytes placed on monolayers of 10^5 L cells to give the indicated Ly:TGC ratios. NRS or antisera (100 μl) were then added as indicated, and the cultures were allowed to incubate at 37°C for 24 hr, after which the remaining adherent L cells were counted as described in *Materials and Methods*. Data are expressed as averages of duplicate determinations with standard error consistently less than 10%.

[b] Experiments 1 to 4: target cells are L-929 treated with mitomycin-C (0.5 μg/ml).

[c] Numbers in parentheses express per cent neutralization of cytotoxicity.

[d] Experiment 5: target cells are growing L-929.

unstimulated human lymphocytes was next investigated. Shown in Table II are the results of 11 different experiments employing anti-α-LT, anti-β-LT, and anti-W.S. antisera. In general, anti-α-LT (B series) antisera are less effective in blocking MICC of PHA-coated L cells when unstimulated lymphocytes are used as effector cells. In only five of 11 experiments was anti-α-LT able to block cytotoxicity to a significant extent. Anti-β-LT did partially neutralize cytotoxicity, and these results were obtained with both soluble Con A- and PHA-coated target cells. Although both anti-α and anti-β antisera partially neutralized MICC, the most consistently effective inhibition was seen with anti-W.S. antisera.

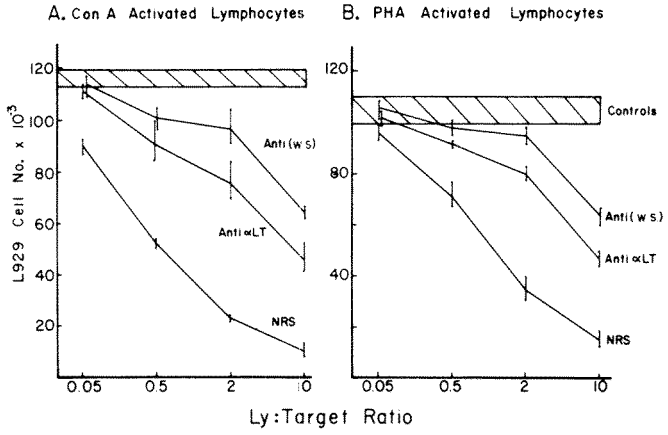

Figure 2. Inhibition of MICC of PHA-coated L-929 TGC by various anti-LT antisera. *A*, Various numbers of Con A (5 μg/ml) preactivated human lymphocytes in 1 ml were added to monolayers of 10^5 mitomycin-C treated L-929 TGC, which were previously coated with PHA-P as described in *Materials and Methods*. Various anti-LT antisera (200 μl) were then added and the cultures allowed to incubate at 37°C for 24 hr. The lymphocytes were then removed, the monolayers washed, and the remaining adherent L cells counted on a Coulter counter. *B*, Same as in *A*, except PHA-P (20 μg/ml) preactivated human lymphocytes were employed as effector cells.

TABLE II

Effect of various anti-LT antisera on MICC L cells by unstimulated human lymphocytes in vitro[a]

Exp.	Ly:TGC	System	% Viability			
			NRS	Anti-α-LT	Anti-β-LT	Anti-W.S.
1	1:1	PHA coated	32	24 (0)[b]		
2	1:1	PHA coated	48	86 (65)		
3	1:1	PHA coated	46	88 (74)		
4	5:1	PHA coated	16	21 (5)		
5	1:1	PHA coated	54	51 (0)	61 (18)	87 (71)
6	5:1	PHA coated	8	44 (22)	68 (48)	56 (40)
7	1:1	PHA coated	42	58 (28)	65 (35)	85 (72)
8	2:1	PHA coated	18	30 (14)	49 (40)	
9	5:1	Con A	14	15 (0)	59 (51)	87 (71)
10	1:1	Con A	45	81 (65)	82 (66)	
11	0.5:1	Con A	30	78 (68)	68 (57)	83 (75)

[a] Human tonsil or adenoid lymphocytes were obtained and cultured overnight at 37°C in MEMS (3% FCS). These cells were then washed, the viability determined by 0.1% eosin Y dye exclusion (routinely >85% viable), resuspended in fresh medium, and various numbers placed on monolayers of 10^5 L cells, which were previously PHA coated. Otherwise, Con A was added as described in *Materials and Methods*. The cultures were then incubated at 37°C for 24 hr, after which the lymphocytes were removed, and the remaining adherent L cells were assayed as described in *Materials and Methods*. Data are expressed as averages of duplicate determination for each point. Standard error is less than 10%.

[b] Numbers in parentheses represent per cent neutralization of cytotoxicity.

Capacity of IgG fractions of anti-LT to block MICC. To identify that the blocking activity of anti-LT sera was due to antibody, IgG fractions were isolated as described in *Methods*. These fractions were also absorbed extensively on unactivated human lymphocytes, L-929 cells, and individual Sepharose 4B affinity columns covalently bound with PHA, human or bovine serum. As can be seen in Table III, the IgG fractions obtained from both anti-α-LT and anti-W.S. antisera were very effective in blocking MICC of L cells *in vitro*.

Effect of anti-LT sera on the response of human lymphocytes to mitogen stimulation. To verify that inhibition of MICC by anti-LT sera was not due to inhibitory effects of these sera on the effector lymphocytes themselves, experiments were performed to assess the effect of these sera on the proliferative response of human lymphocytes to mitogen stimulation measured by thymidine incorporation into DNA. Table IV shows that the 3-day proliferative responses to both Con A and PHA in the presence of IgG fractions are identical in both anti-α-LT IgG or control (NRS) IgG-treated cultures. Additional experiments verified that anti-α-LT IgG does not alter either dose-response curves to these mitogens, responses to mixed lymphocyte stimulation (Table IV), or lymphokine release, e.g., interferon (data not shown). However, proliferative responses to Con A or PHA stimulation were slightly affected in the presence of anti-W.S. IgG. Additional experiments also verified that these antisera are nontoxic to the lymphocytes since overnight incubation at 37°C in 10% antiserum yields viabilities identical to NRS or untreated controls.

Effect of anti-LT antisera on the lymphocyte-independent phase of MICC in vitro. Since it has been reported that lymphocyte-target cell interaction can result in target cell lysis in the absence of the effector lymphocyte (17), we decided to investigate the capacity of anti-LT sera to inhibit cytolysis during the lymphocyte-independent phase of MICC. Preactivated (Con A) human lymphocytes were allowed to interact with PHA-coated target L cells or L cells in the presence of soluble Con A for 30 to 60 min at 37°C, after which the lymphocytes were physically removed as described in *Materials and Methods*, the monolayers were washed, and anti-LT or NRS in fresh MEMS was added. As can be seen in Table V, addition of either anti-α-LT or anti-W.S. after 30 to 60 min of lymphocyte-target cell interaction yields virtually complete protection in both systems. To verify that physical dislodgement removed all aggressor lymphocytes, the following experiments were performed: 1) lymphocytes were not visible by

TABLE III

Inhibition of MICC using anti-LT IgG or Fab fragments[a]

Ly:TGC (+ Con-A)	NRS (IgG) 250 μg	% Viability					
		Anti-α-LT (IgG μg)		Anti-W.S. (IgG μg)		Anti-W.S. Fab (μg)	
		50	250	50	250	200	500
5:1	12	42	83	59	96		
0.5:1	30	53	89	70	94		
1:1	23					39	61

[a] IgG fractions of anti-α-LT, anti-W.S., NRS, or Fab fragments of anti-W.S. were isolated as described in *Materials and Methods*, and the indicated amounts (in 0.1 ml) added to the MICC assay. Soluble Con A was then added (5 μg/ml), and the cultures were allowed to incubate for 24 hr at 37°C. The lymphocytes were then removed, the L cell monolayers washed, and the remaining cells enumerated on a Coulter counter.

TABLE IV

Effect of various antisera on human lymphocyte responses to activation by mitogen or allogeneic stimulation (MLC) in vitro[a]

Treatment	Con-A (μg/ml)			PHA (μg/ml)		MLC		
	0	2	10	0	10	A only	B only	A + B
None	5,600	85,300	61,300	5,500	120,600	1,840	1,250	19,500
NRS, 300 μg IgG	9,500	88,000	69,500	8,900	109,800			14,850
Anti-α-LT, 300 μg IgG	8,500	86,200	60,100	7,000	115,000			16,725
Anti-W.S., 300 μg IgG	33,000	54,500	35,300	26,000	82,000			
BSA, (300 μg)	3,600	84,000	65,000					

[a] Human adenoid lymphocytes were established in MEM (10% FCS) in microplates at 5×10^5 viable cells/well and activated with various concentrations of Con A or PHA as indicated. The MLC reaction was initiated by culturing allogeneic human lymphocytes together in a 1:1 mixture. The IgG fractions of various antisera or NRS were also added as shown. On day 3, lymphocyte proliferation was assayed by pulsing the cells with ^{3}H thymidine (1 μCi/well) for 6 hr at 37°C. The microplates were then processed in a MASH II harvester. Data are expressed (in cpm) as averages of duplicate or triplicate wells for each point with standard error consistently less than 10%.

TABLE V

Effect of various antisera on the lymphocyte independent phase of mitogen induced cytotoxicity of L cells in vitro[a]

	Ly:TGC Ratio		
	10:1	2:1	0.5:1
I. Con A			
NRS	17[b]	23	39
Anti-α-LT	62	75	86
Anti-W.S.	68	88	96
	50:1	20:1	2:1
II. PHA coated TGC			
NRS	45	58	65
Anti-α-LT	92	100	102
Anti-W.S.	88	92	103
Uncoated TGC	96	105	103

[a] Human tonsil or adenoid lymphocytes were established in culture and activated with Con A (5 μg/ml) for 3 days. The cells were then washed with α-MAM, the viability determined, and various concentrations of lymphocytes in MEMS were placed on target L cells in the presence of (I) soluble Con A (5 μg/ml), or (II) PHA-coated L cells. The lymphocytes and L cells were incubated at 37°C for 45 min, after which the lymphocytes were removed by physical dislodgement, the L cell monolayers washed with PBS, 1 ml of fresh MEMS (3% FCS) was added. Various antisera (100 μl/tube) or NRS were then added, and the tubes were allowed to incubate at 37°C for 24 hr. The tube cultures were then assayed as described in *Materials and Methods*.

[b] Per cent target cell viability.

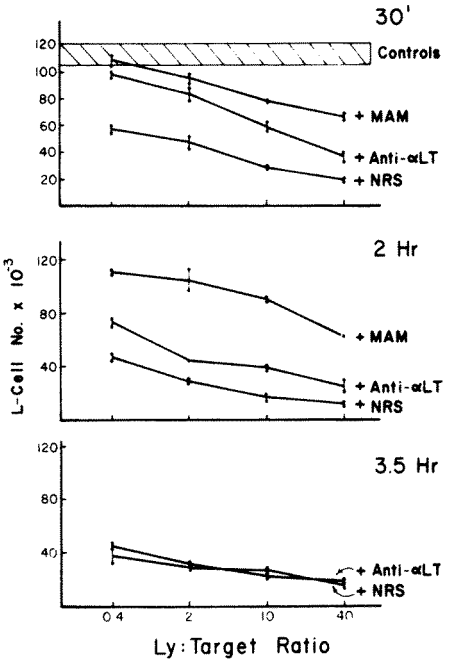

Figure 3. Kinetics of inhibition of lymphocyte independent phase of MICC by anti-LT antisera in vitro. Con A preactivated human lymphocytes (day 3) were washed and placed on target L cells at the indicated Ly:TGC ratios in the presence of fresh Con A (5 μg/ml) or Con A + MAM for 30 min, 2 hr, or 3.5 hr as shown. The lymphocytes were then removed, the monolayers washed with PBS, and NRS or anti-α-LT antisera (100 μl) added for 24 hr at 37°C. The viable adherent L cells were then enumerated by using a Coulter counter as described in *Materials and Methods*.

microscopic observation of target cell monolayers; 2) Coulter counts of trypsinized experimental and control monolayers gave identical cell counts; and 3) treatment of the washed L cell monolayers with rabbit anti-human lymphocyte serum and guinea pig C for 20 min at 37°C gave identical results obtained by removal of the lymphocytes with physical means.

Kinetics of inhibition of the lymphocyte-independent phase of MICC by anti-LT antisera. The lymphocyte independent phase of MICC is inhibited by the presence of anti-LT. We next investigated the duration of the anti-LT-sensitive lymphocyte-dependent phase of cytolysis. Preactivated human lymphocytes (Con A) were allowed to interact with target L cells in the presence of soluble Con A for various periods of time, after which the lymphocytes were removed, various antisera were added, and the target cells were incubated for 24 hr, as described in *Materials and Methods*. As shown in Figure 3, when lymphocytes and target cells are allowed to interact in the presence of Con A for 30 min (30-min lymphocyte-dependent phase), addition of anti-α-LT or anti-W.S. gave good protection, as previously observed. After a 2-hr interaction, addition of anti-LT serum is only slightly less effective in blocking MICC. However, after 3½ hr of interaction, addition of anti-LT serum yields virtually no protection.

DISCUSSION

Human lymphotoxins are a complex "interrelated" family of biologic macromolecules. They can be physically separated into distinct m.w. classes, the major ones termed α and β, and we have developed antisera which will neutralize their cytolytic activity *in vitro*. The antisera employed in the present studies are not directed against single homogeneous proteins but will inactivate different classes or the entire LT family of cytotoxins. Anti-whole supernatant (anti-W.S.) is polyvalent serum, and the only antiserum employed in these studies that will neutralize *in vitro* all the various human LT molecules. This antiserum has reactivity against other lymphokines *in vitro*, i.e., interferon, migration inhibitory factor, and leukocyte inhibitory factor, however, it has been made "lymphokine

specific" by extensive absorption against nonlymphokine proteins present in a supernatant, e.g., PHA, bovine serum, human serum, as well as nonactivated human lymphocyte antigens. The second class of antisera are more specific, that is, they are antisera obtained from animals that were immunized with fractions containing all members of the α-LT class (70 to 90,000 m.w.) or β-LT class (35 to 50,000 m.w.) of cytotoxins. These antisera will functionally distinguish between α and β-LT classes. In addition, within each class of LT molecules there are multiple subclasses which are antigenically distinct, and monospecific antisera have now been developed which can specifically neutralize individual LT molecules. The logic in developing and employing these types of sera was 2-fold: 1) anti-W.S. were used as a screening sera to indicate the potential involvement of LT in a cytodestructive reaction, and 2) more specific antisera were employed to identify specifically the involvement of a particular class or individual subclass in these reactions. This is the first report in a series of studies employing these anti-LT sera to examine the potential role of various LT in lymphocyte-mediated cytodestructive reactions *in vitro*.

The present results reveal that lysis of L cells induced by human lymphocytes in the presence of PHA or Con A *in vitro* is strongly inhibited by these anti-LT antisera. Moreover, the data suggest that different classes of LT molecules can be employed during the cytotoxic reaction(s) since the most effective antisera were the anti-W.S. sera, which will inhibit all LT molecules *in vitro*. Those antisera which inhibit the α and β classes of LT molecules also block cytotoxicity, but to a lesser extent. The present data also suggest that unactivated and preactivated human lymphocytes may employ different classes of LT molecules as lytic effectors. However, this is probably not the case since more recent studies with different antisera indicate all classes of LT molecules are employed by both unactivated and preactivated human lymphocytes during target L cell lysis *in vitro*.

It is of utmost importance to determine the mechanism whereby these sera are able to block mitogen-induced cytotoxic reactions *in vitro*. In the conditions employed in the present experiments, these antisera appear to block only the lytic phase of the CML reaction since they did not: a) affect proliferation to mitogen stimulation; b) affect recognition and proliferation in the MLC reaction; c) interfere with lymphocyte-target cell contact; and d) affect the capacity of stimulated lymphocytes to release other lymphokines, e.g., interferon. In addition, each serum was exhaustively absorbed with unstimulated human lymphocytes, human and bovine serum proteins, and mitogens to remove antibodies that would be directed at nonlymphokine antigens and that could potentially interfere with CML reactions. However, we found that absorption of anti-LT sera with activated lymphocytes removed all blocking and LT-neutralizing activity. These studies, to be published in another manuscript, revealed that LT molecules are expressed on the surface of activated lymphocytes (31, manuscript in preparation). In addition, each serum has also been chemically fractionated to contain only IgG immunoglobulins (identified by both double diffusion and immunoelectrophoresis) and finally, any involvement of Fc fragments in blocking cytolysis has been excluded by employing Fab fragments obtained from certain antisera. Altogether, these studies strongly support the concept that the blocking effects of these antisera are mediated by antibody active sites binding to LT molecules. This concept is also supported by studies which showed that LT activity could be specifically bound and recovered from Sepharose affinity columns to which these same antisera were covalently linked (32).

It appears that antiserum blocking of cell-mediated lysis occurs during the lymphocyte-independent phase of the cytotoxic reaction(s). This is clearly shown in studies in which the lymphocyte was allowed to interact with the target cell (lymphocyte-dependent phase), then physically removed, and the antisera subsequently were added. These studies rule out any possibility that the antisera are affecting the aggressor lymphocyte. Furthermore, these antisera totally blocked lysis of L cells during the lymphocyte-independent phase, when mediated by both Con A- and PHA-activated lymphocytes. However, kinetic studies revealed these antisera could only block lysis for a brief period of time during the first 3 hr of the LT-target cell interaction. These two observations indicate that during this time interval, LT either induces an irreversible lesion to the cell, loses its antigenicity, or physically enters the cell and is no longer accessible to the neutralizing action of antibody. These data collectively support the concept that, in this system, the lymphocyte deposits cytotoxic LT effector molecule(s) on the surface of the L cell during the lymphocyte-dependent phase, which can mediate destruction of that target cell, even in the absence of the effector lymphocyte.

Lysis of target cells by mitogen-activated lymphocytes is indeed a complex *in vitro* phenomenon. It has been shown by employing both murine and human effector lymphocytes, that T cells mediate lysis of allogeneic target cells, whereas T and B cells may both mediate lysis of xenogeneic target cells *in vitro* (33). There is a report that Fc-bearing cells (null?) in these populations may also mediate lysis of target cells when mitogen stimulated, as a result of antibody produced by B cells, which is reactive with the target cell (34). The effector cells in this xenogeneic aggressor-target cell system are both T and B (manuscript in preparation). Clearly, both types of effector cells are blocked by these antisera. There are at least two possible mechanisms or pathways by which an effector T or B lymphocyte could destroy a xenogeneic cell in the presence of a mitogen. One involves a mandatory contact with the target cell membrane and implicates a direct membrane lytic system. The other involves a loose or transient interaction with the target cell and may involve a local secretory delivery system. Gately *et al.* (3), studying immune guinea pig effector lymphocytes on xenogeneic target cells, have proposed that these two systems involve different cytolytic mechanism(s). We approached the question of contact-mediated cytotoxicity *vs* a secreted effector molecule with the PHA-coated (contact required) *vs* soluble Con A-induced (contact not absolutely required) cytotoxic systems. Our data suggest that the lysis which occurs when there is close contact between aggressor lymphocyte and target L cell (PHA coated) is a much more difficult reaction to block with anti-LT sera (more antisera are required) than lysis induced by addition of Con A. However, both appear to be mediated by the same mechanism(s), since contact-mediated cytotoxicity (PHA coated) is exquisitely sensitive to the action of anti-LT antibody during the lymphocyte-independent phase after the contact between these cells has been interrupted. This implies that the arrangement of the cells during contact probably restricts the ability of antibody to penetrate into the local microenvironment due to steric hindrance in the contact region. This observation suggests that, under these conditions, LT is either placed directly on the target cell surface or is effectively secreted, only at these contact points, inaccessible to the effects of antisera. A highly localized delivery mechanism(s), perhaps mediated by mem-

brane contact, is supported by the finding of membrane-associated LT molecules (31). These data indicate that it is possible for LT to be involved in both types of lytic mechanism, contact or secretion, depending on the type of lymphocyte involved or nature of lymphocyte-target cell interaction.

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