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Identification of Membrane-Associated Lymphotoxin (LT) on Mitogen-Activated Human Lymphocytes Using Heterologous Anti-LT Antisera *in Vitro*[1]

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Surface-associated lymphotoxin (LT) molecules have been identified on mitogen-activated human lymphocytes employing heterologous anti-α-LT serum *in vitro*. These membrane-associated LT molecules are present on PHA- or Con A-activated lymphocytes but do not appear to be expressed on unstimulated cells. Furthermore, these molecules were detected primarily on activated T lymphocytes, with little detectable on activated B- or null-cell populations. The removal of surface LT-bearing lymphocytes, using anti-α-LT serum + C', does not dramatically affect the capacity of the remaining cells to release LT after mitogen restimulation. In addition, the presence of toxic molecules on the surface of activated lymphocytes suggests that these materials may be expressed in an inactive, noncytotoxic form.

INTRODUCTION

In a previous publication, we found that lysis of L cells by mitogen-stimulated human lymphocytes *in vitro* could be significantly inhibited using heterologous anti-lymphotoxin (LT) antisera (1). However, during these experiments we found that when anti-LT sera were absorbed on activated lymphocytes (to remove potential antibodies against lymphocyte surface antigens), almost all *in vitro* blocking and LT neutralizing activity of the sera was removed. However, absorption of these same antisera on unstimulated lymphocytes had no apparent effect. This observation suggested that LT molecules may be expressed on the surface of activated lymphocytes *in vitro* with little or none detectable on unstimulated lymphocytes. The finding that LT can exist on activated lymphocyte surfaces is a new concept, for in the past, LT has *only* been considered as a "soluble-phase" mediator (2–6). This conclusion has grown from experiments which show that LT release (7), as well as MIF release (8), is suppressed by agents which inhibit protein synthesis or secretory processes (9) or alter levels of cyclic nucleotides (cAMP) (10).

This manuscript presents the results of further study of surface-associated LT on human lymphocytes using heterologous anti-α-LT antisera *in vitro*. In addition, the

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relationship of membrane-associated LT to the classical concept of LT as a soluble-phase mediator is discussed.

MATERIALS AND METHODS

Target Cells and Culture Medium

Stock cultures of a sensitive strain of mouse L-929 fibroblasts (α-L-929) were used as target cells (11). These cells were maintained in 32-oz prescription bottles in 95% air, 5% CO_2, and passed biweekly. Culture medium consisted of minimal essential medium with Hanks salts, supplemented with 3% heat-inactivated (56°C, 60 min) fetal calf serum (FCS) (Microbiological Associates, Bethesda, Maryland), 0.2 $\mu g/ml$ of glutamine, penicillin (100 U/ml), and streptomycin (100 $\mu g/ml$) (MEMS).

Lymphocyte Cultures

Suspensions of human small lymphocytes from tonsils or adenoids were obtained within 6–8 hr of surgical removal from normal children, as previously described (7). Lymphocyte suspensions were adjusted to 4×10^6 viable cells/ml in 32-oz prescription bottles, using MEMS, and incubated for 5–6 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, Michigan) at 20 $\mu g/ml$ or concanavalin A (Con A, Sigma, St. Louis, Missouri) at 5 $\mu g/ml$. Nonstimulated lymphocytes were cultured in an identical fashion, except mitogens were omitted. When employed as effector cells, PHA-activated lymphocytes were pipetted vigorously to break up clumps and washed 2× with MEMS before use. To those lymphocytes activated with Con A, a competitive binding inhibitor of Con A, α-methyl-D-mannoside (α-MAM), was added to 5 mM, and the cells were vigorously pipetted and then washed 2× in MEMS plus 5 mM α-MAM to remove all residual cell-bound Con A.

Separation of T and B Lymphocytes

Lymphocytes were obtained from adenoids as already described, washed twice with MEMS, and mixed with a 12-fold excess of washed sheep erythrocytes (sRBC) in 100% filtered FCS. This mixture was centrifuged at 300g for 8 min, and the pelleted cells were allowed to incubate for 1 hr at 4°C. T-cell rosettes were then separated from free cells by density-gradient sedimentation through Ficoll–Hypaque ($p = 1.090$). The interface cells from this procedure were termed (B + null) cells, and the pellet was termed T cells. The purity of these cell populations was determined by fluorescence staining using rabbit anti-human Ig serum. This procedure routinely yields 90–95% T cells and 93–97% (B + null) cells. These separate populations were then cultured in MEMS containing 20 $\mu g/ml$ of PHA-P for 5 days at 37°C. The cells were then collected and washed twice with 40 ml of MEMS. The final cell pellet was then used for antisera absorption as described in Methods.

Lymphotoxin Assay

Details of this assay have been previously described. Briefly, target L-929 cells (10^5 cells in 1 ml) were established as monolayers in screw-capped tubes in MEMS

containing 0.5 μg/ml of mitomycin C. After 24 hr at 37°C, the medium was discarded, the monolayers were washed with 0.01 M phosphate, 0.15 M NaCl, pH 7.12) (PBS), and the remaining adherent cells, were trypsinized and enumerated using a Model F Coulter counter. Units of LT activity per milliliter are determined by the reciprocal of the dilution necessary to destroy 50% of the target L cells.

Direct Lymphocyte-Mediated Cytotoxicity

Target L-929 cells (10^5 cells in 1.0 ml of MEMS containing 0.5 μg/ml of mitomycin C) were established 24 hr prior to use, identical to the LT assay. The medium was discarded, the monolayers were washed with PBS, and 1 ml of a cell suspension containing a predetermined number of lymphocytes was added to each tube. Concanavalin A (Con A) was then added to each tube at 5 μg/ml, or Con A plus 5 mM α-MAM, a competitive binding inhibitor of Con A, were 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 cells were then removed by trypsin treatment and counted, identical to the LT assay.

Preparation of Anti-LT Antisera

Human LT molecules can be separated into several distinct classes based on their molecular weight, termed complex (greater than 150,000), α (70,000–90,000), β (25,000–50,000), and γ (12,000–20,000) (13, 14). The α and β classes can be further fractionated into subclasses by ion exchange chromatography and electrophoresis (15, 16).

The methods for generating and testing various rabbit anti-human LT sera have been reported previously (17–19). Sera employed in the present study were of two types: (a) anti-α-class LT and (b) and anti-whole supernatant. Anti-α-class serum was obtained from rabbits immunized with Sephadex fractions containing all α-LT subclasses, but free of all other classes. This antiserum will neutralize the lytic effect of all human α-LT subclasses in vitro. Anti-whole supernatant (WS) was obtained from animals immunized with fresh protein-free supernatants from lectin-activated human lymphocyte cultures. These latter sera are the only sera which will neutralize in vitro all presently identified human LT classes and subclasses. Control sera were collected from these animals before immunization with various preparations or from normal unimmunized rabbit serum (NRS). All sera were heat-inactivated (56°C for 45 min) and filter-sterilized before use.

Preparation of Absorbed IgG Fractions

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 a 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 3.4×20-cm DEAE-cellulose column. The fractions containing IgG were collected, pooled, concentrated by ultrafiltration, and dialyzed against PBS. Double-diffusion and im-

munoelectrophoresis revealed that the preparation contained only IgG. Each sample (100 mg) was then incubated with 1.4×10^9 human lymphocytes/ml for 12 hr at 4°C, after which the cells were removed by centrifugation. Five to ten milligrams per milliliter each of PHA-P, bovine serum, and human serum was linked covalently to Sepharose 4B by the method of March et al. (20). Each serum sample was then passed sequentially over individual 10-ml columns of Sepharose 4B linked to one of the above proteins. Double-diffusion analysis employing the above materials revealed no bands of precipitation after the IgG fractions were passed over the column.

Absorption of Antiserum on Separated and Unseparated Mitogen-Activated Human Lymphocytes

Mitogen (Con A)-activated (3–6 days) or unstimulated human lymphocytes or separated lymphocyte subpopulations were chilled to 4°C and washed twice (50 ml each) with cold MEMS. The lymphocyte pellet was then resuspended in anti-α-LT serum and allowed to incubate for 1 hr at 4°C with occasional mixing. The cells were removed by centrifugation for 10 min at 900g, and the absorbed serum was collected. To determine whether the absorption had removed LT neutralizing capacity from the anti-α-LT serum, various amounts (0–200 μl) of the absorbed antiserum were added to a preparation containing approximately 100 units of human α-LT activity in MEMS, and incubated at 37°C for 1 hr. The amount of LT activity remaining in the test and control samples was then determined.

Lysis of Activated Human Lymphocytes Using Anti-α-LT Serum and Complement (C′)

Human lymphocytes were cultured for various intervals with or without Con A and washed twice with cold MEMS as described in the previous section. These lymphocytes were subsequently resuspensed in a density of 10^7/ml in MEMS containing various dilutions of either anti-α-LT, anti-WS, anti-lymphocyte serum (ALS), or NRS, and incubated for 1 hr at 4°C with occasional mixing. The cells in this suspension were washed once with 10 ml of cold MEMS and resuspended in 1 ml of MEMS containing a 1:20 dilution of guinea pig serum as a source of complement (C′). After 1 hr at 37°C, the cells were washed once, using 40 ml of PBS, and the total viable cell number was determined in 0.1% eosin Y using a Neubauer counting chamber or intact cells were enumerated in a Model F Coulter counter.

Preparation and Use of ^{125}I-Labeled Goat Anti-Rabbit IgG (GARIgG)

Goat anti-rabbit IgG (GARIgG) was obtained from hyperimmune goat serum from animals immunized with rabbit IgG. The IgG fraction of this antiserum was applied to a Sepharose 4B affinity column containing bound rabbit IgG. The GARIgG was then eluted with two column volumes of acid buffer, 0.1 M acetate, pH 2.4, neutralized with NaOH, dialyzed against PBS, and finally labeled with ^{125}I by the immobilized lactoperoxidase method of Thorell and Larsson (21). The ^{125}I-labeled GARIgG was stored at -20°C until used. Double-antibody labeling of human lymphocytes was performed as follows: Mitogen preactivated or unstimulated human adenoid lymphocytes were collected, chilled to 4°C, and washed twice with cold PBS

containing 0.5% bovine serum albumin (BSA), 0.02% Na azide, pH 7.2 (PBS-BSA). Lymphocytes (4×10^6/ml) were suspended in 1 ml of PBS-BSA, and 100 μl of anti-α-LT or NRS was added, then allowed to incubate for 1 hr at 4°C. The lymphocytes were then washed twice with PBS-BSA and resuspended in 1 ml of PBS-BSA containing 5 μl of ^{125}I-labeled GARIgG. This mixture was then allowed a further incubation of 1 hr at 4°C, and washed twice with PBS-BSA. Lymphocyte-bound ^{125}I-labeled GARIgG was then determined by counting in a Beckman gamma counter (Beckman Industries).

Preparation and Use of Membrane-Enriched Subcellular Fraction (MEF) Obtained from Human Lymphocytes in Vitro

Mitogen-preactivated or unstimulated human adenoid lymphocytes were collected after 5 days at 37°C, chilled to 4°C, washed with MEMS, and homogenized at 4°C, using a rotary cell homogenizer (dounce homogenizer). After 95% cell lysis (verified by microscopic observation), the homogenate was spun at 400*g* to remove whole cells and nuclei. The membrane-enriched fractions (MEF) were poured off and washed 2× with cold PBS, and the protein concentration was determined by absorption at 280 and 260 nm. Various concentrations of MEF were then incubated with target L cells for 24 hr at 37°C. After this interval, the L-cell monolayers were washed with PBS, and the remaining viable adherent L cells were enumerated in the Coulter counter as described for the LT tube assay.

RESULTS

Removal of the Capacity of Anti-α-LT Antiserum to Block α-LT-Mediated Lysis of L Cells by Absorption on Mitogen-Preactivated Human Lymphocytes in Vitro

Heterologous antiserum directed against the stable 70,000–90,000 MW class of α-lymphotoxins was developed in rabbits as described in Methods (anti-α-LT). The antiserum effectively inhibited cytotoxicity mediated by Sephadex G-150 fractions containing only human α-LT when tested on L-929 target cell *in vitro* (Fig. 1, left). However, when 1.5 ml of anti-α-LT antiserum is absorbed on 2×10^8 4-day mitogen-preactivated human lymphocytes for 1 hr at 4°C, its capacity to block α-LT-induced lysis of L cells *in vitro* is greatly reduced (Fig. 1, right). This effect is a dose-related phenomenon, since absorption on a larger number of mitogen-preactivated lymphocytes (6×10^8) yields greater specific antibody removal. This reduction in LT neutralizing capacity is not due to the presence of additional LT which is released into the antiserum during the absorption process, since NRS when absorbed under the same conditions is nontoxic to L cells. In contrast, this effect is not seen when anti-α-LT serum in absorbed on a similar number of unstimulated human adenoid lymphocytes (Fig. 1, right) or on nonlymphoid cells, such as human HeLa or mouse L cells (data not shown). This suggested that LT molecules may be expressed on the surface of activated human lymphocytes but not on unstimulated lymphocytes.

Specific Removal of Anti-α-LT Activity by Absorption of Anti-LT Serum on T or B + Null Lymphocyte Subpopulations

To determine which population of human lymphocyte was responsible for the selective removal of anti-α-LT antibody, adenoid lymphocytes were separated into

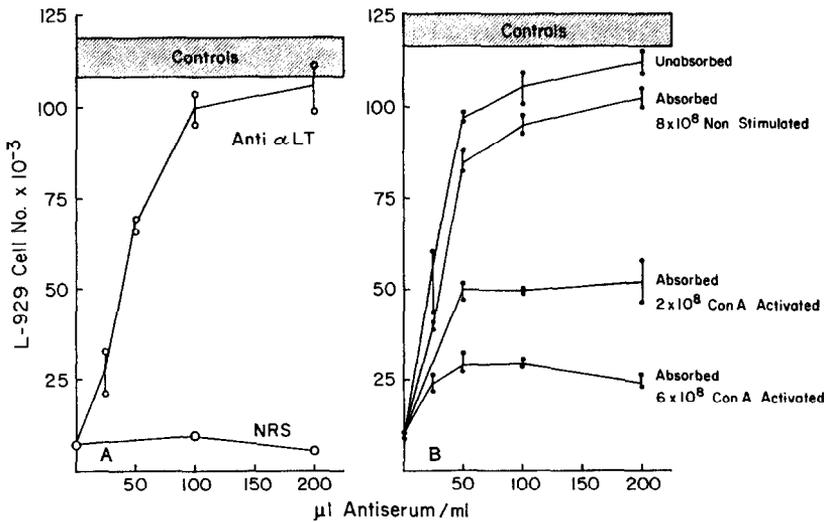FIG. 1. Removal of anti-α-LT antibody by absorption on mitogen-preactivated human lymphocytes *in vitro*. (Left) To 100 units of human α-LT (in 1 ml of MEMS), various amounts of rabbit anti-α-LT antiserum or NRS were added and allowed to incubate for 1 hr at 37°C. This mixture was then added to L-929 target cells for 24 hr at 37°C. The remaining viable adherent L-929 cells were then enumerated on a Coulter counter. (Right) Anti-α-LT antiserum (1.5 ml) was incubated for 1 hr at 4°C with various numbers of nonstimulated or Con A-activated (Day 4) human lymphocytes. Various amounts of this absorbed antiserum (shown on abscissa) were then incubated with 100 units of α-LT in 1 ml of MEMS for 1 hr at 37°C, and the residual α-LT activity was tested on L-929 target cells for 24 hr at 37°C. The remaining viable adherent L-929 cell number was then determined on a Coulter counter.

T and B + null cell subpopulations as described in Materials and Methods. These cell populations were then cultured in MEMS and activated with 20 μg/ml of PHA-P or 5 μg/ml of Con A for 5 days. The cells were then collected, prechilled, washed with MEMS, and tested for their capacity to selectively absorb anti-α-LT antibody. As can be seen in the data presented in Fig. 2, 4×10^7 cells from a 93% T-enriched population completely removed the neutralizing capacity in 0.5 ml of anti-α-LT serum. In contrast, a similar number of B + null cells was much less effective. In addition, similar data have been obtained employing human peripheral blood lymphocyte subpopulations activated with either PHA or Con A *in vitro*.

Identification of Membrane-Associated LT Using Anti-α-LT Serum and Radiolabeled Second Antibody

To further identify the presence of surface-associated LT molecules ^{125}I-radiolabeled goat antibody directed against rabbit IgG (GARIgG) was used. For these experiments, mitogen-preactivated or unstimulated (Day 5) human lymphocytes were collected, washed, and suspended in anti-α-LT serum or NRS (100 μl/4 × 10^6 cells) for 1 hr at 4°C. The cells were again washed and resuspended in PBS-BSA containing ^{125}I-labeled GARIgG, as described in Materials and Methods. Shown in Table 1 is a representative result of four such experiments. As can be seen, low levels ^{125}I binding (cpm ^{125}I bound/10^7 cells) are obtained when both stimulated and unstimulated lymphocytes are labeled with ^{125}I-labeled GARIgG without prior incubation with anti-α-LT antiserum. However, after prior incubation

in anti-α-LT serum, mitogen-preactivated lymphocytes show a significant increase (approximately fourfold) in ^{125}I labeling compared to unstimulated lymphocyte controls.

In Vitro Lysis of Mitogen-Preactivated Human Lymphocytes Using Anti-α-LT Serum, Anti-WS Serum, and C′

The capacity of various anti-LT serum to mediate C′-dependent lysis of mitogen-preactivated or unstimulated human lymphocytes *in vitro* was investigated. Mitogen (Con A, PHA)-preactivated or unstimulated lymphocytes were collected 5 days post stimulation, chilled to 4°C, washed, and suspended in a 1:20 dilution of anti-α-LT, anti-WS, anti-lymphocyte serum (ALS), or NRS for 1 hr at 4°C. The cells were then washed and suspended in a 1:20 final dilution of guinea pig complement (C′). After 1 hr at 37°C, the remaining viable cells were counted in a Neubauer chamber. As shown in Fig. 3, treatment of Con A-preactivated lymphocytes with anti-α-LT serum + C′ killed approximately 25% of the cell population, while treatment with anti-WS + C′ killed about 40% of the cell population. As a positive C′ control, ALS + C′ was effective in destroying 85–95% of these cells. In contrast, when unstimulated lymphocytes are employed, treatment with either anti-α-LT or anti-WS + C′ causes very little reduction over NRS or untreated controls.
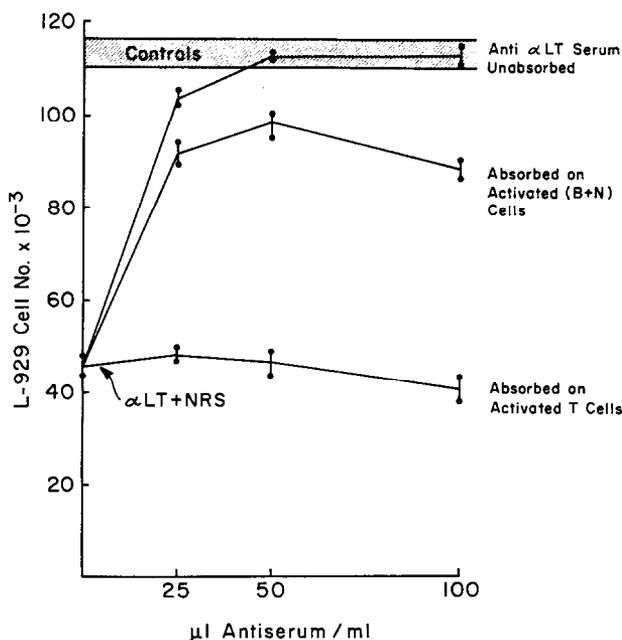

FIG. 2. Removal of anti-α-LT antibody by absorption on mitogen-preactivated human T- or B + null-lymphocyte subpopulations *in vitro*. Anti-α-LT antiserum (0.5 ml) was incubated for 1 hr at 4°C with 4×10^7 mitogen-activated human T- or B + null-lymphocyte subpopulations separated from adenoids as described in Materials and Methods. Various amounts of this absorbed serum (shown on abscissa) were then incubated with 30 units of α-LT in 1 ml of MEMS for 1 hr at 37°C, and the residual α-LT activity was tested on L-929 target cells for 24 hr at 37°C. The remaining viable adherent L-929 cell number was then determined on a Coulter counter.

TABLE 1

Double-Antibody Labeling of Mitogen-Preactivated or Unstimulated Human Lymphocytes Using Rabbit Anti-α-LT Antiserum Plus ^{125}I-Labeled Goat Anti-Rabbit IgG (GARIgG)[a]

Lymphocytes	Anti-α-LT (100 μl)	^{125}I-Labeled GARIgG	[125]I Bound (cpm/10^7 cells)
Unstimulated	—	—	250 (background)
Unstimulated	—	+	800 ± 70
Unstimulated	+	+	2000 ± 150
Activated	—	+	750 ± 100
Activated	+	+	7400 ± 380

[a] Mitogen-activated or unstimulated human adenoid lymphocytes were collected after 5 days, washed, and duplicate samples were incubated with anti-α-LT serum as described in Materials and Methods. They were then washed out of the antiserum and resuspended in 1 ml of PBS-BSA containing 5 μl of ^{125}I-labeled GARIgG. After 1 hr at 4°C, the cells were washed, and the cell pellet containing the bound ^{125}I-labeled GARIgG was counted in a Beckman gamma counter.

Additional studies found that C'-mediated lysis of preactivated lymphocytes using various anti-LT sera does not appear to be due to passive absorption of LT onto the membrane. Since unstimulated lymphocytes, when incubated in 500 units of soluble LT at 37°C for 1 hr, chilled to 4°C, and washed two times, are not lysed by treatment with anti-LT + C.

Capacity of Absorbed Anti-α-LT IgG to Cause C'-Dependent Lysis of Mitogen-Preactivated or Unstimulated Lymphocytes in Vitro

To determine if the C'-dependent lysis of mitogen-preactivated lymphocytes was due to antibodies in the anti-α-LT serum, which might be directed at lymphocytes surface-associated components other than LT, these antisera or IgG fractions were extensively absorbed. They were exposed to unstimulated human lymphocytes and passed through individual Sepharose 4B columns with bound PHA, bovine, and human serum proteins. The ability of absorbed anti-α-LT and anti-WS IgG to cause C'-mediated lysis of mitogen-preactivated human lymphocytes is shown in Table 2. Clearly, anti-α-LT IgG + C' can still cause lysis of mitogen-activated, but not unstimulated, human lymphocytes. About 25% lysis is obtained with absorbed anti-α-LT IgG +C', while approximately 35% lysis occurs using absorbed anti-WS IgG + C'.

Effect of Anti-α-LT Serum + C' on Direct MICC and LT Release in Vitro

The next experiments were performed to examine the capacity of lymphocytes to cause mitogen-induced lysis (MICC) of L-929 target cells or to release LT upon mitogen restimulation after treatment with anti-α-LT serum + C'. Five-day Con A-preactivated lymphocytes were treated with a 1:20 dilution of anti-α-LT, anti-WS (which had been absorbed as described in Materials and Methods), ALS, or NRS. After 1 hr at 4°C, the cells were washed and resuspended in a 1:20 dilution of guinea pig C'. After 1 hr at 37°C, the cells were washed twice, counted, and placed in L-929 target cells with 5 μg/ml of Con A at various lymphocyte: target cell ratios. As can be seen in Fig. 4, MICC is partially reduced, but not totally ablated after treatment with anti-α-LT IgG + C'. ALS is also quite effective
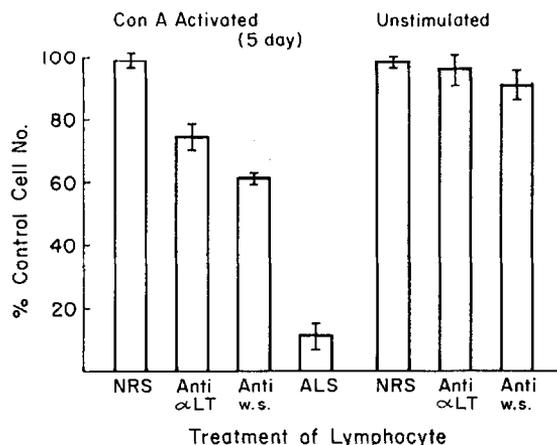
Con A Activated
(5 day)   Unstimulated

FIG. 3. Complement (C')-mediated lysis of mitogen-preactivated human lymphocytes using anti-α-LT antisera *in vitro*. Mitogen-stimulated (5-day) or unstimulated human adenoid lymphocytes were collected, washed with PBS containing 5 mM α-methyl-D-mannoside to remove cell-bound Con A, and suspended in a 1:20 dilution of NRS, anti-α-LT, anti-whole supernatant (anti-WS), or anti-lymphocyte serum (ALS), as indicated. After 1 hr at 4°C, the cells were again washed with PBS and resuspended in a 1:20 final dilution of guinea pig complement (C'). After 1 hr at 37°C, the remaining viable cells were enumerated by microscopic observation of the cells suspended in 0.1% eosin Y solution in a Neubauer chamber. Control cell numbers were obtained by counting cells which were untreated or treated with C' only.

in removing the capacity of these cells to cause MICC *in vitro*. In contrast, the capacity of unstimulated lymphocytes to cause MICC after treatment with either anti-α-LT or NRS + C' is not affected. To investigate whether lymphocytes could release LT after treatment with anti-α-LT + C', 5-day Con A-stimulated lymphocytes were treated with anti-α-LT + C', washed, counted, and recultured in fresh MEMS containing 5 μg/ml of Con A for 3 days. After this time, the supernatants were obtained and tested for LT activity as described in Materials and Methods. Shown in Table 3 are the results of a representative experiment of three such experiments. Clearly, while treatment of stimulated lymphocytes with anti-α-LT or anti-WS + C' removes about 20–30% of the cells, the levels of LT released by

TABLE 2

Complement-Mediated Lysis of Con-A-Activated or Unstimulated Human
Lymphocytes Using Absorbed Anti-α-LT IgG *in Vitro*[a]

Treatment	Percentage control	
	Con A activated	Unstimulated
Untreated	100	100
NRS IgG (100 μg) + C'	98 ± 2	100
Anti-α-LT IgG (100 μg) + C'	74 ± 3	98 ± 1
Anti-WS IgG (100 μg) C'	65 ± 3	86 ± 2

[a] Identical procedures were employed as described in Fig. 3, except that anti-α-LT and anti-WS IgG were obtained by DEAE-cellulose chromatography and absorbed on unstimulated human lymphocytes (1.4 × 10⁹/100 mg of IgG) and affinity columns of PHA and human and bovine serum proteins, as described in Materials and Methods.
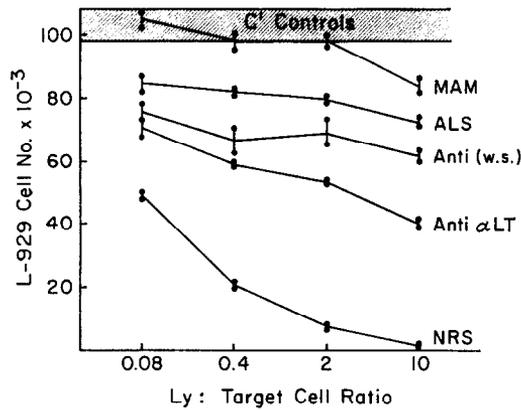
FIG. 4. Removal of cytotoxic effector cells using anti-α-LT + C' *in vitro*. Con A-activated human lymphocytes (4 day) were collected, washed with PBS containing 5 mM α-methyl-D-mannoside (MAM), and treated with anti-α-LT, anti-WS, ALS, or NRS and complement (C'), as described in Fig. 2. Various numbers of the remaining lymphocytes were then placed on 10^5 L-929 target L cells in the presence of 5 μg of fresh Con A or Con-A + a competition binding inhibitor of Con A, MAM, as controls. After 24 hr at 37°C, the remaining viable adherent L-929 cells were enumerated on a Coulter counter.

the remaining lymphocytes are comparable to those of untreated or NRS-treated controls.

In Vitro Destruction of L Cells by Isolated Membrane-Enriched Fractions (MEF) Obtained from Mitogen-Preactivated Lymphocytes

Mitogen-preactivated or unstimulated lymphocytes (5 day) were collected and washed, and the membrane-enriched fractions (MEF) were isolated as described in Materials and Methods. Various concentrations of MEF were then placed on L-929 target cells with or without anti-α-LT antiserum and incubated at 37°C for 24 hr. As can be seen in the data presented in Table 4 MEF obtained from 5-

TABLE 3

Release of LT *in Vitro* by Con-A Restimulation of Residual Human Lymphocytes after Treatment with Anti-α LT Sera + Complement (C')

Treatment after 5 days in culture[a]	Percentage destruction	Restimulation with Con A	LT activity detected after 72 hr at 37°C
None	0	—	2 ± 0
None	0	+	41 ± 2
NRS + C'	1.5	+	39 ± 4
Anti-α-LT + C'	26	+	31 ± 3
Anti-α-LT IgG + C'	19	+	39 ± 4
Anti-WS + C'	24	+	32 ± 3

[a] Human adenoid lymphocytes were stimulated with 5 μg/ml of Con A in MEMS for 5 days. They were then collected, washed, treated with anti-α-LT + C' as described in Materials and Methods, counted, and recultured at 2 × 10^6 ml with or without 5 μg of Con A in fresh MEMS for an additional 72 hr at 37°C. Lymphocyte viability at the end of this time was routinely >70%. The supernatants were collected and tested for LT activity, as described in Materials and Methods.

TABLE 4

Destruction of L-929 Target Cells by Isolated Membrane-Enriched Subcellular Fractions (MEF) of Activated and Unstimulated Human Lymphocytes *in Vitro*[a]

Preparation	Percentage L-cell viability
Expt. 1.	
Con-A-activated intact lymphocytes (5:1)	11 ± 6
MEF from activated lymphocytes (1 mg)	34 ± 4
MEF from activated lymphocytes (0.1 mg)	83 ± 5
MEF from unstimulated lymphocytes (1 mg)	97 ± 3
Supernatant from final MEF-activated wash (undiluted)	86 ± 3
Expt. 2.	
MEF from unstimulated lymphocytes (1 mg)	96 ± 1
MEF from activated lymphocytes + 100 μl of NRS	29 ± 4
MEF from activated lymphocytes + 100 μl of anti-α-LT	100 ± 4

[a] Con-A-activated or unstimulated human lymphocytes were obtained after 5 days, and the membrane-enriched fraction (MEF) was isolated as described in Materials and Methods. The protein content of the MEF was determined by absorbance at 260 and 280 nm. Various concentrations of MEF were then placed on 10^5 mitomycin C-treated L cells with or without 100 μl of anti-α-LT antiserum for 24 hr at 37°C. After this time, the remaining viable adherent L cells were enumerated on a Coulter counter.

day Con A-activated human lymphocytes effectively destroys target L cell *in vitro*. In addition, anti-α-LT antiserum (100 μl) can significantly inhibit MEF-mediated lysis. In contrast, MEF obtained from unstimulated lymphocytes when employed at the same concentrations was ineffective at causing L-cell destruction.

DISCUSSION

In vitro studies have revealed that the human LT system is composed of multiple classes of molecules which can be separated one from another by physical–chemical methods (13–16). Within each class of LT molecules are various subclasses which can also have different antigenic specificities (17, 25). Two different types of rabbit antisera or IgG fractions which will neutralize human LT-induced cell lysis *in vitro* were employed in the present studies. The first antiserum was obtained from rabbits immunized with Sephadex fractions containing all of the stable α subclasses (MW, 70,000–90,000) of human LT cytotoxins. The capacity of these sera to neutralize α-class LT molecules *in vitro* has been reported in other publications (17, 18, 23). While this antiserum is not directed against a single purified α-LT subclass, it has limited reactivity, for it does not interact with other human lymphokines *in vitro*, i.e., MIF, LIF, CF, or LT molecules from other animal species (25). In addition, studies to be presented elsewhere have revealed that immunoprecipitation of radiolabeled crude lymphocyte supernatants using absorbed anti-α-class serum or IgG fractions yields two major bands of labeled proteins on SDS gels. A second antiserum has also been developed which will neutralize *all* currently known human lymphotoxin molecules *in vitro*, as well as a variety of other lymphokines which are present in whole supernatants (e.g., interferon, MIF, LIF). This antiserum was obtained from rabbits immunized with fresh

whole supernatants, and thus is a polyvalent serum, but has been rendered "lympho-kine specific" by extensive absorption against nonlymphokine proteins which might be present in a lymphocyte supernatant, e.g., lectins, human and bovine serum, as well as nonactivated human lymphocyte antigens.

The present results indicate that LT molecules are present on the surface of lectin-activated human lymphocytes *in vitro*. Within the limits of our ability to detect these materials, they do not appear to be expressed on unstimulated lympho-cytes *in vitro*. Several criteria support his finding: (a) The capacity of anti-α-LT serum to neutralize soluble α-LT-induced destruction of L cells *in vitro* could be specifically removed when these sera were absorbed with stimulated but not unstimulated lymphocytes. Activated human T-lymphocyte cell populations were the most effective in binding anti-LT immunoglobulins. (b) Activated human lympho-cytes were sensitive to C'-dependent lysis using anti-α-LT serum, while unstim-ulated cells were resistant. (c) Use of ^{125}I-labeled goat anti-rabbit IgG could detect higher levels of rabbit anti-α-LT bound to the surface of activated lymphoid cells when compared to unstimulated controls. And finally (d) crude, membrane-enriched fractions (MEF) could destroy L cells *in vitro* if derived from activated human lymphocytes. All of these experiments were performed at 4°C to minimize degradation, pinocytosis, or phagocytosis of surface-associated antibodies. The toxicity of these MEF, while 50- to 100-fold less effective than the intact lympho-cyte, appears to be due to membrane-associated LT, since washing could not remove lytic activity; and anti-α-LT sera blocked the MEF toxicity.

It seems unlikely that the lymphocyte surface components recognized by these two anti-LT sera are not LT but other material(s). Although the rabbit anti-α-LT serum was made against fractions which could have contained PHA, we avoided the possibility of anti-PHA antibody by absorbing the antiserum against Sepharose-bound PHA and by activating human lymphocytes with a non-cross-reacting mitogen, Con A. In addition, complement-mediated lysis, using Con A-activated lymphocytes, gave identical results to those obtained when the lymphocytes were activated with PHA. Extensive absorption of the anti-α-LT and anti-WS sera on unstimulated human lymphocytes reduced the possibility that these effects are due to antibodies directed against lymphocyte surface proteins which might cochrom-atograph with α-LT (especially histocompatibility antigens). This point is especially relevant, since it has been shown by McCune *et al.* that the density of HLA antigens expressed on activated human lymphocytes *in vitro* increases as much as 10-fold after stimulation with PHA (24). In addition, specific removal of the LT neutralizing capacity of anti-α-LT serum by absorption on activated, but not on unactivated, human lymphocytes further negates the probability that C'-mediated lysis is due to anti-histocompatibility antibodies present in this serum. Absorption of these sera on affinity columns of Sepharose 4B, covalently bound to bovine and human serum proteins, also reduces the possibility of antibodies in these sera reactive against these antigens. Finally, a previous study showed that incubation with anti-α-LT neither alters the human lymphocyte proliferative response to Con A or PHA stimulation, nor affects recognition or proliferation in the MLC reaction *in vitro* (1). This antiserum does not appear to affect normal lymphocyte physiological responses or functions.

The identification of LT molecules associated with T-lymphocyte surface mem-branes raises the question of why the lymphocyte does not destroy itself, since

soluble-phase human LT has been considered to be a "nonspecific" cytotoxin. These findings indicate that the lymphocyte bearing these surface LT molecules must either (a) be resistant, or (b) express them in such a manner that they are not active. This situation is being investigated; however, there are several possibilities. Surface-associated LT molecules may have directional orientation such that the lytically active site(s) of the molecules are directed away from the cell. This would imply that these substances have nontoxic membrane receptor sites separate from lytically active regions. Alternatively, membrane-associated LT molecules may be expressed in a lytically inactive form, and require a steric or enzymatic modification to become active. The stimulus to induce these materials to become lytically active must also be associated with membrane receptors which recognize antigens and which can be activated by interaction with lectins. Once activated, LT molecules may be shed or released from the cell surface. This latter concept is supported by the findings that, once on the cell surface, lectins can induce their release in the complete absence of protein synthesis and secretion (23). The physical nature of surface-associated LT molecules is as yet unknown, however, they are the topic of intensive study.

Lysis of target cells by mitogen-activated lymphocytes is a complex *in vitro* phenomenon. It has been shown, employing both murine and human effector lymphocytes, that in the presence of PHA or Con A, T cells mediate lysis of allogeneic target cells, whereas T and B cells may both mediate lysis of xenogeneic target cells *in vitro* (27). The effector cells in the xenogeneic aggressor–target L-cell system employed in our studies are both T and B cells (manuscript in preparation). However, we found that both types of effector cells are blocked by various classes of anti-LT sera (1). The removal of surface LT-bearing lymphocytes (T cells), as well as the observation that the residual (nonsurface LT-bearing) B lymphocytes can release LT *in vitro*, suggest that contact of target cells with lymphocytes bearing LT on their surface and secretion of LT are both effective pathways for target-cell lysis in this system. These findings are relevant to the proposed mechanisms of T cell-mediated cytotoxicity *in vitro*. Gately *et al.* have suggested that direct lymphocyte-mediated destruction of target cells *in vitro* occurs via two mechanisms, one involving LT (as a soluble mediator) and one involving intimate contact between lymphocytes and target cells (28). It has also been reported by Huber *et al.* (29) that LT-releasing murine lymphocytes bear Ly 1+ antigenic markers, whereas cytotoxic T lymphocytes bear Ly 2, 3 + surface markers. The present studies imply that these may be T lymphocytes capable of expressing LT on their surface without necessarily releasing it into the soluble phase. Thus, the concept that within subsets of murine T lymphocytes there are two distinct populations of cells which appear to either secrete lymphokines or function as cytotoxic killer lymphocytes presupposes that cytotoxic killer lymphocytes could not express LT molecules on their surface. In the same logic pattern, Ferluga and Allison reported that isolated membrane-enriched fractions obtained from activated Balb/C mouse spleen cells could destroy ^{51}Cr-labeled P815 mastocytoma cells *in vitro*, while unstimulated murine lymphocyte membrane fractions were not active (22). These investigators argued that the mechanism of cytolysis caused by these isolated membrane fractions was independent of the action of LT, since LT is known only as a secreted soluble-phase effector molecule. We have shown in the present study that MEF isolated from activated human lymphocytes can destroy

target L cells *in vitro*, and that this cytolysis is inhibited by anti-α-LT antisera. Thus, LT molecules could be involved in a number of different cytolytic mechanisms as soluble-phase or membrane-associated effectors.

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