Title

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Journal

Cellular Immunology, 41(2)

ISSN

0008-8749

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Publication Date

1978-12-01

DOI

10.1016/0008-8749(78)90235-6

CELLULAR IMMUNOLOGY 41, 380–396 (1978)

The Human LT System

IV. Studies on the Large MW LT Complex Class: Association of these Molecules with Specific Antigen Binding Receptor(s) *in Vitro*

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Received April 17, 1978

The present studies demonstrate that a portion of lymphotoxin (LT) cell-lytic activity present in supernatants from: 1) lectin (Con A, PHA) stimulated nonimmune; or 2) antigen (soluble or cellular) stimulated immune human lymphocytes *in vitro,* is associated with immunoglobulin (Ig) or "Ig-like" receptor molecule(s). This concept was supported by three findings: 1) LT activity in these supernatants was partially inhibited by heterologous anti-human (IgG) Fab'_2 antisera; 2) LT activity present in soluble antigen stimulated immune human lymphocyte supernatants could specifically bind to and be eluted from Sepharose 4B columns to which the specific stimulating antigen was covalently attached; and 3) LT activity present in primary one-way mixed lymphocyte culture (MLC) supernatants could be removed by absorption on the specific stimulator cells. The amount of total LT activity found to be associated with "Ig" in these supernatants was variable, but ranged from 5 to 20% in lectin stimulated cell supernatants to 20 to 50% in antigen or MLC stimulated supernatants. Physical-chemical studies on the molecular weight class of LT molecules having reactivity with anti-Fab'_2 sera, as well as antigen binding capacity, revealed these properties reside in the large (> 200,000) MW LT class, termed complex. The nature and biological significance of these "antigen specific" LT complexes, as they relate to mechanisms of cytotoxicity *in vitro,* will be discussed.

INTRODUCTION

Lymphocytes are important effector cells involved in various forms of cellular immune tissue destructive reactions both *in vivo* and *in vitro*. Clearly, these reactions are complex, for they involve different classes of effector lymphocytes and different types of *in vitro* experimental test systems (1–4). Moreover, lysis of target cells in these various test systems can be rapid or protracted, or highly specific or nonspecific. While our understanding of the initial step(s) in these reactions is becoming clear, the biochemical processes involved in the actual lytic events are still not clear. It has been proposed that target cell lysis may involve the synthesis and release of a "nonspecific soluble" cell toxin(s), termed lymphotoxin (LT) (5). While the role of LT in these various cell lytic reactions is controversial, there is evidence accumulating to merit support of the above concept. This has largely derived from studies employing antisera which will neutralize certain forms of

0008-8749/78/0412-0380$02.00/0

soluble LT activity *in vitro* (6, 7). However, it is still not clear how, at the molecular level, "nonspecific soluble" toxins may participate as lytic effectors in highly specific *in vitro* cytotoxic reactions, unless their effects are highly localized and directed by the aggressor lymphoid cell(s).

Human LT molecules are physically and immunologically a heterogeneous system of biological macromolecules (8–10), which can exist in both soluble and membrane associated forms (11, 12). These materials can presently be resolved into four basic MW classes, termed complex (> 200,000 Daltons), α (70–90,000 D), β (35–50,000 D), and γ (10–25,000 D), and several of the classes resolved into multiple subclasses by differences in molecular charge (10, 13). Our recent findings revealed that these various soluble LT classes and subclasses represent a "system" of molecules which can associate with one another to form the large MW complex class (14, 15). Initial studies also suggested that these complexes could further associate with immunoglobulin (Ig) molecules. This finding suggested these molecules could have antigen binding capacity if derived from an antigen stimulated immune cell(s). This concept, if substantiated could have important implications to reveal a specific mechanism(s) of delivery of a "nonspecific" cell-toxin(s) to a target cell by an aggressor lymphocyte(s).

The present manuscript reports the results of further studies on the human LT system and reveals findings which suggest that cytotoxic activity (LT) present in supernatants of antigen stimulated immune human lymphocytes *in vitro* can: 1) associate with Ig molecules; 2) once associated with Ig, these LT molecules possess specific antigen binding capacity; and 3) this antigen binding capacity resides in the large MW LT complex class of lymphocyte released cell toxins. The relationship of these Ig associated "antigen specific" LT complexes, as they relate to the mechanism(s) of cellular cytotoxicity *in vitro,* will be discussed.

MATERIALS AND METHODS

1. *Target Cells and Culture Media*

Target cells were obtained from stock cultures of mouse L-929 fibroblasts maintained in RPMI-1640 (Grand Island Biological Co. (GIBCO), Grand Island, N.Y.), supplemented with 3% heat inactivated (56°C, 1 hr) fetal calf serum (FCS) (GIBCO), 100 U/ml penicillin, and 100 μg/ml streptomycin (RPMI-S). These cells were grown at 37°C in 32 oz prescription bottles in 95% air, 5% CO_2, and passed biweekly. Suspension cultures of the continuous human lymphoblastoid cell lines, RPMI-1788, WI-2 (both B cell lines), and Molt-4 (a T cell line) were also maintained in RPMI-1640, but supplemented with 10% FCS.

2. *Lymphocyte Cultures and Supernatants*

Human lymphocytes employed for these studies were obtained from two sources: 1) tonsils and adenoids, as previously described (16); or 2) peripheral blood. Peripheral blood lymphocytes were obtained from normal human donors previously skin tested for delayed sensitivity to a panel of test antigens, including SKSD and tetanus toxoid. Mononuclear cells were isolated after density gradient centrifugation on Ficol–Hypaque ($\rho = 1.077$). The interface cells were collected, washed, and suspended in RPMI-S to a density of 2×10^6/ml. These cells were routinely 90 to 95% lymphocytes and 95 to 100% viable. The lymphocytes were then acti-

vated by addition of either: 1) specific antigen (streptokinase-streptodornase (SKSD) (Lederle, Pearl River, N.Y.) (200–300 U/ml approximately 10–15 μg protein/ml), or tetanus toxoid (TT) (Lilly, Indianapolis, Indiana) (1 μg/ml); 2) the lectins concanavalin A (Con A) at 5 μg/ml, or phytohemaggluinin-P (PHA-P) at 20 μg/ml; or 3) mitomycin C treated allogeneic lymphocytes at a 10:1 responder:stimulator ratio (MLC). After 5 to 7 days at 37°C, the lymphocytes were removed by centrifugation, and the cell free supernatants were collected, and immediately assayed for LT activity or frozen at − 70°C until used. The levels of LT activity present in these supernatants were variable, and ranged from 10 to 50 units/ml (antigen stimulation), 5 to 25 units/ml (MLC stimulation), and 100 to 400 units/ml (lectin stimulation). When used for column chromatography, the supernatants were routinely concentrated 10 to 20 times by ultrafiltration, using Amicon PM-10 membranes.

3. *LT Assays*

Lymphotoxin (LT) was assayed in crude or concentrated supernatants by determining the percent survival of mitomycin C treated target cells, as previously reported (17). Briefly, mouse L-929 cells were established as monolayers in 16 × 125 mm screw-capped glass tubes at a concentration of 10^5 in 1 ml RPMI-S, in the presence of 0.5 μg/ml mitomycin C. Following overnight incubation at 37°C, the media was poured off, and serial dilutions of LT containing or control media in RPMI-S were added. The tubes were then incubated for 24 hr at 37°C, after which the dead cells were poured off in the supernatant, and the remaining viable adherent cells were trypsinized and enumerated in a Model F Coulter Counter. One unit of LT activity is defined as the amount necessary to cause 50% destruction of 10^5 viable cells following a 24 hr incubation. The amount of LT activity in units/ml in a given supernatant is determined by the reciprocal of the greatest dilution which kills 50% of the target cells.

Fractions from gel filtration or affinity columns were tested for LT activity by adding 100 to 200 μl of each fraction to duplicate 1 ml tube cultures of L-929 cells for 24 hr at 37°C. The viable adherent cell number was then determined identical to the LT assay previously described.

4. *Preparation and Use of Antigen Affinity Columns*

Protein antigens, SKSD, tetanus toxoid, or BSA, were covalently coupled to CNBr activated Sepharose 4B by the method of March, *et al.* (18). Three milliliters of beads containing 1 mg/ml protein antigen were packed into 5 ml syringes and equilibrated in 0.15 *M* NaCl, 0.01 *M* phosphate, pH 7.2 (PBS). Supernatants obtained from antigen or lectin activated human lymphocytes were concentrated 10 times by ultrafiltration on Amicon PM10 membranes, and 2 ml were slowly passed into the columns and allowed to incubate for 30 to 60 min at 25°C. Afterwards, the columns were washed with 20 ml PBS, and 4 ml 2 *M* KI in PBS were then slowly eluted through the columns. The fractions containing KI were dialyzed against 1000 volumes PBS for 24 hr at 4°C. All fractions were then tested for cytotoxic activity as described above. The cytotoxic elution profiles were plotted on graph paper, and the nonbinding and binding peaks were then cut out and weighed to determine the percent of LT activity in each peak.

5. *Preparation and Use of Anti-Fab'$_2$, Anti-WS and Anti-α-LT Antisera*

The methods for production of immunogens, immunization, and specificity of these antisera have been previously reported (9, 19–20).

A. *Anti-Fab'$_2$.* Fab'$_2$ fragments were prepared from normal human IgG molecules by pepsin digestion, according to methods previously described (19). Antisera were obtained after repeated immunization of New Zealand white rabbits with Fab'$_2$ suspended in CFA.

B. *Anti-whole supernatant (Anti-WS) and anti-α_2-LT serum.* Production and characterization of these antisera has been previously reported (9, 20). *Anti-WS:* Briefly, a goat was immunized by the method of Vaitukaitus (21) with whole unfractionated supernatants obtained from lectin (PHA-P) activated human lymphocytes in serum free media. This antisera has been shown to effectively neutralize *all* presently known classes and subclasses of soluble human LT molecules *in vitro* (9, 20). *Goat anti-α_2-LT serum:* The precise details for generation and the reactivity of this antisera has been described in another manuscript (9). Briefly, Sephadex G-150 fractions containing the 70 to 90,000 D α-LT activity peak were pooled, concentrated by ultrafiltration (PM-30), and rechromatographed on Sephadex G-150. This method has been shown to yield functionally purified α-LT preparations free of other MW LT classes (10, 22). These α-LT preparations were further subjected to fractionation by ion exchange chromatography on DEAE, as previously described (10). A linear elution salt gradient (0–0.3 *M* NaCl) was employed, and fractions corresponding to the α_2-LT subclass eluting between 0.05 to 0.15 *M* NaCl were pooled, concentrated, emulsified in CFA, and used to immunize a goat. While this sera was made against a highly refined α_2-LT fraction, it is a *very* potent serum and reactive with various forms of LT molecules. The anti-α_2-LT sera was tested by double diffusion analysis against whole human serum or intact human IgG molecules and revealed no visible bands of precipitation. In addition, this sera and its IgG fraction were passed through immunoabsorbants (Sepharose 4B) containing covalently linked human and bovine serum. This treatment did not affect the LT neutralizing capacity of this antiserum.

C. *Anti-human Heavy Chain Sera.* Various goat anti-human heavy chain specific antisera (anti-γ, anti-μ, and anti-γ, μ, δ) were obtained from Cappel Laboratories (Cochranville, PA). These antisera were dialyzed against 1000 volumes PBS at 4°C before use.

All sera were heat inactivated (56°C – 1 hr) and centrifuged 20,000*g* for 30 min before use. Neutralization of LT activity by these various sera was performed by the addition of 20 to 100 μl of antiserum, or normal goal serum (NGS), to 1 ml of supernatant, preincubating for 1 hr at 4°C, and then testing for remaining LT activity on L-929 cells. Percent neutralization of LT activity in a given supernatant by these antisera is determined as follows:

$$\% \text{ Neutralization} = \frac{(\text{Units LT Act/ml} + \text{NRS}) - (\text{Units LT Act/ml} + \text{Antisera})}{(\text{Units LT Act/ml} + \text{NRS})} \times 100.$$

6. *Gel Filtration Chromatography*

Degassed Ultrogel AcA 44 (LKB, Upsala, Sweden) was poured to a bed height of 90 cm in 2.5 × 100 cm siliclad coated glass columns and equilibrated with 100

TABLE 1

Inhibition of Lectin or Antigen Induced LT Activity by Heterologous Rabbit Anti-Human FAB'$_2$, Anti-WS, or Anti-α_2-LT Antisera *in Vitro*[a]

Lympho-cyte donor	Agent used to induce LT	*NRS*	% Neutralization of LT activity by					
			Anti-WS	Anti-α_2-LT	Anti-Fab-$_2$	Anti-Fab-$_2$ (IgG Fx)[b]	Anti-Fab'$_2$ (absorbed)[c]	Anti-oval
I.S.	SKSD	2 ± 1	94 ± 5	92 ± 5	32 ± 4	27 ± 4	4 ± 1	1 ± 1
	TT	3 ± 1	96 ± 4	96 ± 7	29 ± 6	30 ± 4	2 ± 1	—
	Con A	1 ± 1	98 ± 2	94 ± 3	12 ± 2	10 ± 3	2 ± 1	3 ± 1
G.G.	SKSD	0	95 ± 5	93 ± 6	31 ± 4	30 ± 5	2 ± 1	2 ± 1
	Con A	1 ± 1	97 ± 5	98 ± 2	14 ± 5	—	2 ± 2	—

[a] Supernatants from lectin (Con A) or antigen (SKSD, TT) stimulated human peripheral blood mononuclear cells were collected 5 to 7 days post-stimulation, cleared of cells, and tested for reactivity with rabbit anti-Fab'$_2$ sera, goat anti-WS sera, or anti-α_2-LT serum, as described in Methods.

[b] The IgG fraction of the anti-Fab'$_2$ antisera was obtained from chromatography on DEAE-cellulose of a 40% $(NH_4)_2$ SO_4 fraction of whole serum. Two hundred and fifty micrograms of IgG in 50 μl PBS was employed for these experiments.

[c] The IgG fraction of the anti-Fab'$_2$ antisera was incubated with either whole human IgG or Fab'$_2$ fragments for 2 hr at 37°C, and then 6 hr at 4°C. The resulting precipitate was removed by ultracentrifugation at 20,000 rpm for 1 hr at 4°C, and 50 μl tested for its LT neutralizing activity. In addition, double diffusion analysis revealed this antisera showed no bands of precipitation against IgG or Fab'$_2$ after incubation with those antigens.

m*M* phosphate buffer, 10^{-4} *M* EDTA, pH 7.2. The columns were calibrated with various molecular weight markers applied to the column in a total volume of 1.5 ml. Six milliliter fractions were collected and monitored for their absorption at 280 nm. Two ml of lymphocyte supernatant, which was concentrated 25 to 50 times by ultrafiltration using Amicon PM10 membranes, were then applied to these columns, and 50 to 200 μl of the eluted fractions were assayed for toxic activity on L-929 cells, as described above.

RESULTS

1. *Inhibition of Lectin or Soluble Antigen Induced Human LT Activity by Heterologous Anti-human (IgG) Fab'$_2$, Anti-WS, or Anti-α-LT Antiserum in Vitro*

During an initial series of experiments, we noticed that a small, but consistent, percentage of LT activity present in lectin (PHA, Con A) activated human lymphocyte supernatants was inhibited by heterologous rabbit antisera directed at the Fab'$_2$ region of normal human IgG molecules (15). Additional experiments revealed inhibition of LT activity by this antisera was generally between 5 to 18%, and varied, depending upon the individual lymphocyte donor or the particular supernatant employed (Table 1). Neutralization of LT activity by anti-Fab'$_2$ sera was due to antibody, since purified IgG fractions also yielded the same level of neutralization. In addition, prior incubation of the anti-Fab'$_2$ antisera with homologous antigen (e.g., Fab'$_2$ fragments) or with intact human IgG molecules removed the LT neutralizing capacity of this antiserum. In contrast, neither normal rabbit

serum (NRS) nor rabbit anti-ovalbumin serum had any effect on the toxic activity in these supernatants. Inhibition of LT activity by anti-Fab'$_2$ or its IgG fractions was also noted with supernatants obtained from antigen stimulated immune human lymphocytes *in vitro*. However, inhibition in this case was usually higher, i.e., 20 to 30%. Table 1 shows that lymphocyte donors I.S. and G. G. (who are skin test positive to both SKSD and TT) produced supernatant toxic activity after stimulation with soluble antigens (SKSD or TT) or lectins (Con A), which was inhibitable by anti-Fab'$_2$ antisera. Furthermore, that this observed toxic activity was due to LT molecules is strongly supported by the finding that cytolysis was essentially completely inhibited by heterologous goat anti-whole supernatant antiserum (anti-WS), or anti-α_2-LT serum, both potent inhibitors of soluble LT activity *in vitro* (9, 20). There is, however, a remote possibility that the toxic activity present in these supernatants is due to classical antibody. However, since the physical properties of LT molecules are quite different from classical Ab molecules, and since the goat anti-α_2-LT serum employed in these studies does not react with human serum proteins or intact human Ig molecules detectable by double diffusion analysis or immunoabsorption, it is highly unlikely that inhibition of the toxic activity is due to interaction of this antisera with Ig molecules. Furthermore, the data presented in Table 2 demonstrates that Ab molecules present in the 50% $(NH_4)_2\ SO_4$ fraction of whole serum from an SKSD or TT sensitive human donor (G.G.) are not toxic to L cells at levels (10% serum or 10 mg of the globulin fraction), which are 500 to 5000 times higher than the amounts of Ab molecules present in these culture supernatants. Thus, these findings suggest that LT molecules can associate with Ig or Ig-like receptor molecules bearing Fab'$_2$ determinants.

2. *Capacity of Soluble Antigen to Stimulate Immune Lymphocytes to Release in Vitro LT activity Which Will Selectively Bind to Specific Antigen Bound to Sepharose 4B Columns*

To test the concept that LT molecules could associate with Ig-like receptors, giving them antigen binding capacity, LT toxic activity present in supernatants obtained from antigen stimulated immune human peripheral blood lymphocytes

TABLE 2

Toxic Activity of Whole Human Serum or the Globulin Rich $(NH_4)_2\ SO_4$ Fraction on L-929 Cells *in Vitro*

Culture condition	Amount used	% cell viability
Human serum	2% (1.3 mg)	100
Human serum	10% (6.5 mg)	88
Human serum $(NH_4)_2\ SO_4\ F_x$	1 mg	100
Human serum $(NH_4)_2\ SO_4\ F_x$	5 mg	100
Human serum $(NH_4)_2\ SO_4\ F_x$	10 mg	98
Human serum $(NH_4)_2\ SO_4\ F_x$	25 mg	67

Serum was collected from donor G.G. (skin test positive to TT and SKSD), heat inactivated (56°C for 1 hr), and various amounts tested for its toxic activity on L-929 cells during a 24 hr incubation at 37°C. The same serum was slowly brought to 50% $(NH_4)_2\ SO_4$, at 4°C, and the resulting globulin rich precipitate collected by centrifugation (20,000*g*, 30'), resuspended in PBS, dialyzed against 1000 volumes of PBS at 4°C, and then tested for toxic activity on L-929 cells. Protein concentration was determined by absorbence at 260/280 nm.

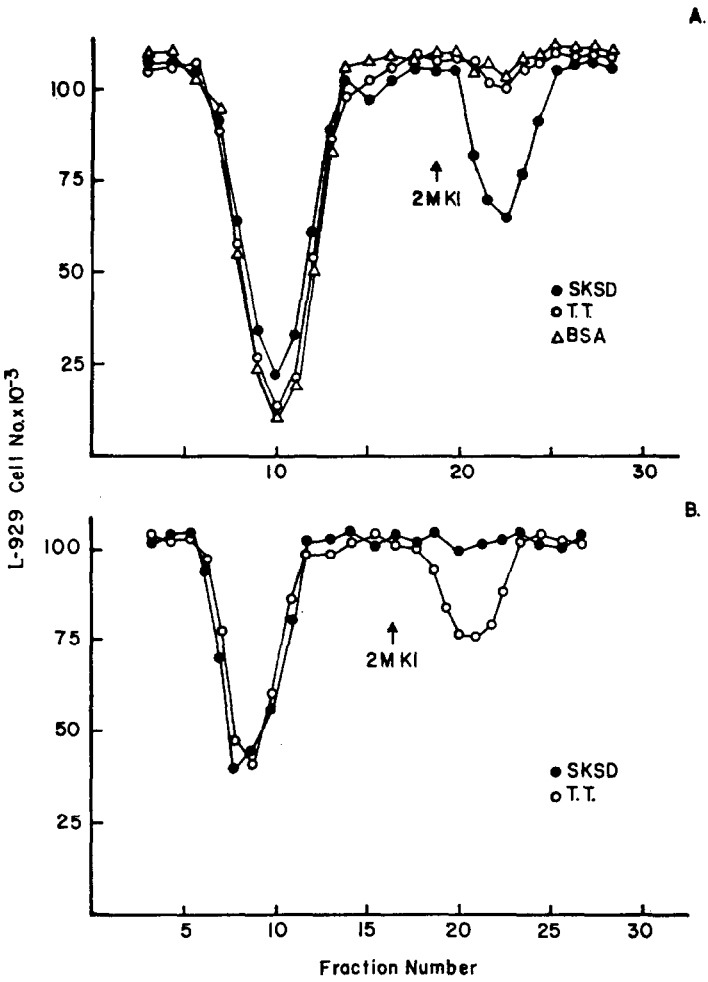

FIG. 1. Elution profile of LT activity present in antigen stimulated immune human lymphocyte supernatants after chromatography on affinity columns to which various protein antigens are covalently attached. (A) Elution profile of LT activity present in a 6 day SKSD induced human lymphocyte supernatant over Sepharose 4B containing SKSD, TT, or BSA covalently attached. Two milliliters of a 10 times concentrated supernatant was eluted through 3 ml of sepharose beads, and the bound LT activity was then eluted with 2 *M* KI. The fractions containing KI were dialyzed against PBS, and the toxic profile was determined by testing 200 μl on L-929 cells as described in Methods. (B) Same as in A, except a 5 day tetanus toxoid (TT) induced human lymphocyte supernatant was eluted through sepharose beads containing either TT or SKSD covalently attached.

in vitro was tested for its capacity to selectively bind to and be eluted from affinity columns to which specific antigen was covalently attached. Shown in Fig. 1 are the results of two experiments. Clearly, LT activity present in supernatants induced after 6 day stimulation of immune human lymphocytes with SKSD can bind to and be eluted from affinity columns to which SKSD is covalently attached (Fig. 1A). The data shown in Table 3 indicate approximately 35% (26–44% in three experiments) of the total LT activity present in these supernatants will bind to affinity columns to which SKSD is covalently attached. Furthermore, this binding ap-

peared to be immunologically specific, since SKSD induced LT activity would not bind to control tetanus toxoid (TT) or BSA affinity columns. In contrast, a significant amount of LT activity (> 29%) present in supernatants of TT stimulated immune lymphocytes will bind only to TT affinity columns and not SKSD columns (Fig. 1B). Furthermore, stimulation of immune lymphocytes with Con A in the absence of antigens (for 5 days) results in virtually no detectable levels of specific antigen binding (SKSD or TT) LT molecules (Table 3). We feel this may be due to the polyclonal stimulatory nature of Con A resulting in only low or undetectable levels of LT-Ig complexes possessing any one antigen specificity. Finally, the percent recovery of total LT activity from these various columns was high, ranging from 78 to 93%.

3. *Specific Inhibition of Antigen Induced LT Cytotoxic Activity by Incubation with Soluble Antigens and Target L Cells in Vitro*

If LT molecules could associate with Ig-like receptor(s), and bind to immobilized antigen, then it seemed possible to attempt to inhibit LT-lytic activity by preincubation with soluble antigen, perhaps leading to formation of microantigen-antibody-LT complexes. These complexes would presumably reduce the total amount of soluble LT activity available to interact with susceptible target L cells, and thus reduce the total amount of detectable LT activity. To test this concept, supernatants obtained from antigen (SKSD or TT) stimulated immune human

TABLE 3

Percentage of Lectin or Soluble Antigen Induced Human LT Activity Which can Selectively Bind to and Be Eluted from Sepharose Beads to Which Specific Protein Antigen is Covalently Attached[a]

Human lymphocyte donor	Agent used to induce LT	% LT activity bound to specific antigen beads		
		TT	SKSD	BSA
I.S.[b]	TT	*36 ± 9*	9 ± 6	5 ± 5
	SKSD	5 ± 5	*33 ± 9*	2 ± 2
	Con A	5 ± 5	5 ± 5	5 ± 2
G.G.[c]	TT	*39 ± 6*	3 ± 2	—
	SKSD	4 ± 2	*33 ± 5*	3 ± 1
C.W.[d]	SKSD	6 ± 1	*30 ± 2*	2 ± 2
	Con A	6 ± 1	3 ± 1	2 ± 1

Error values indicate the range of error in replicate experiments.

The range of error in a single experiment never exceeds 10%.

[a] Supernatants from lectin (con A) or antigen (SKSD, TT) stimulated human peripheral blood mononuclear cells were collected 5 to 7 days post-stimulation, concentrated 10 times, and passed over individual 3 ml affinity columns to which SKSD, TT, or BSA were covalently attached. The bound material was then eluted with 2 *M* KI in PBS and daialyzed against PBS to remove the KI. Toxic activity of the various fractions were then assayed, and elution profiles similar to those shown in Fig. 1 were plotted. The percent of LT activity binding to specific antigen beads was calculated, as described in Methods.

[b] Collective results of three such experiments

[c] Results of two such experiments.

[d] Results of a single experiment.

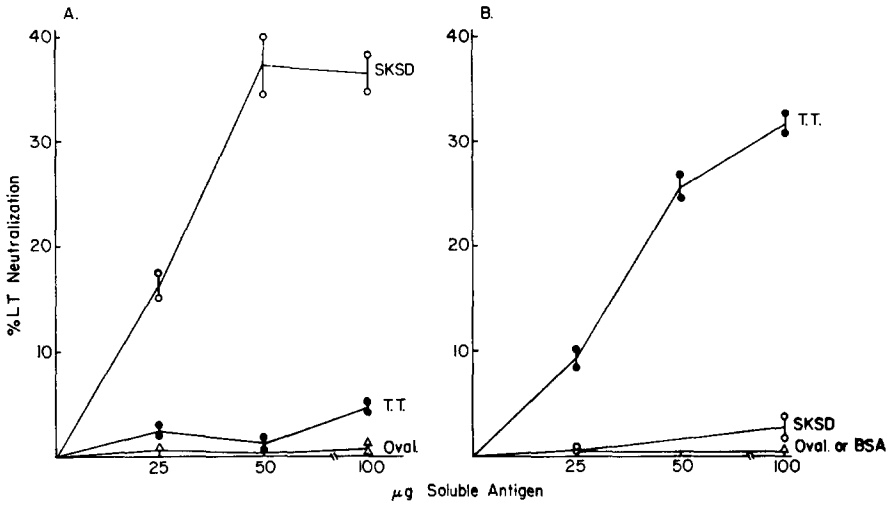

FIG. 2. Inhibition of LT activity present in antigen stimulated immune human lymphocyte supernatants by pre-incubation with soluble antigen. (A) Supernatants obtained after 6 days stimulation of immune human peripheral blood lymphocytes with SKSD were preincubated with various amounts of soluble SKSD, TT, or ovalbumin in PBS for 1 hr at 37°C, and the remaining toxic activity determined on L-929 cells as described in Methods. (B) Same as in A, except supernatant LT activity was obtained by stimulation of immune human lymphocytes with tetanus toxoid (TT).

lymphocytes were preincubated with various amounts of soluble SKSD, TT, or control antigens for 1 hr at 4 or 37°C, and dilutions subsequently tested for remaining lytic activity on L-929 target cells *in vitro*. Shown in Fig. 2 are the representative results of seven such experiments. Clearly, preincubation of SKSD induced supernatants with soluble SKSD can reduce the amount of LT activity detectable in these preparations. Inhibition was a dose related effect but plateaued at a level of approximately 38% reduction at 50 μg/ml SKSD protein. This was very close to the percent of SKSD induced LT activity which would bind to specific SKSD antigen affinity columns (Fig. 1A and Table 3) or was inhibited by anti-Fab'$_2$ antisera *in vitro* (Table 1). This then seemed to represent a simple method for determining the amount of "antigen specific" LT activity in a whole supernatant. Furthermore, inhibition of LT activity by soluble antigen appeared to be immunologically specific, since other protein antigens, including TT, BSA, or ovalbumin would not inhibit SKSD induced LT activity. In addition, inhibition of LT activity by soluble SKSD was not apparently due to nonspecific mechanisms such as contaminating protease activity, etc., because soluble SKSD had no effect on either TT induced LT activity, shown in Fig. 2B, or lectin (Con A, PHA) induced LT activity (data not shown). Furthermore, incubation of TT induced LT activity with soluble TT resulted in approximately 30% inhibition of the lytic activity.

4. *Selective Absorption of MLC Induced Human LT Activity on Specific Stimulator Target Cells in Vitro*

To test the concept that lymphocytes which respond to primary MLC stimulation *in vitro* could release LT molecules in association with material(s) having specificity

for the stimulating cellular antigens, the following experiments were performed. Human adenoid or peripheral blood mononuclear cells were obtained and mixed at a 10:1 ratio with various mitomycin C treated human lymphoblastoid cell lines (RPMI-1788, WI-2, Molt 4) and cultured for 5 to 7 days at 37°C. The supernatants were then collected, and immediately exposed to various numbers of specific sensitizing or control target cells for 1/2 to 1 hr at 4°C, *in vitro*. After incubation, the cells were removed by sedimentation, and supernatants were tested for remaining LT activity on L-929 cells. Shown in Table 4 are the pooled results of several such experiments. Clearly, a significant amount of the total LT activity from 25 to 60% can be removed by absorption on the specific primary MLC stimulater cell *in vitro*. Removal of LT activity was dosage dependent with maximal absorption occurring at approximately 50×10^6 absorbing cells/2 ml supernatant. In contrast, only small amounts (< 10%) are removed when these same supernatants are absorbed on nonrelated allogeneic or xenogeneic (i.e., L-929) target cells *in vitro*. Furthermore, LT activity present in unrelated soluble antigen or lectin induced supernatants was not significantly removed by incubation with these cells. In addition, LT activity present in these various human MLC supernatants was inhibitable (up to 20–40%) by rabbit anti-FAb'_2 serum, compared to normal rabbit serum controls (data not shown). This level of inhibition of LT activity in MLC supernatants by anti-FAb'_2 sera correlates strongly with the level of LT activity removed by absorption on the specific stimulating target cells.

5. *Identification of the Molecular Weight Class of LT Molecules Associated with Antigen Binding Ig-Like Receptors*

Experiments were next designed to determine the molecular weight class of LT molecules possessing the antigen binding characteristics. To do this, human lymphocytes sensitive to SKSD were obtained and cultured for 6 days in the presence of 10 μg/ml soluble SKSD, the supernatants were collected, immediately concentrated

TABLE 4

Specific Removal of LT Activity Present in MLC Induced Human Lymphocyte Supernatants by Absorption with Specific Stimulator Cells *in Vitro*[a]

Responder lymphocyte	Stimulating agent	% neutralization of LT activity by absorption on								
		Autologous lymphocytes	RPMI-1788		WI-2		MOLT 4		L-929	
		5×10^7	10^7	5×10^7	10^7	5×10^7	10^7	5×10^7	10^7	5×10^7
JCH (PBL)[b]	RPMI-1788	3[c]	*37*	*46*	11	12	2	8	3	8
	WI-2	5	7	8	*39*	*62*	6	10	2	9
	Molt-4	—	10	8	—	—	*26*	*39*	—	—
	SKSD	—	9	13	10	14	—	—	—	—
	Con A	—	12	18	9	16	—	—	—	10
Adenoids	RPMI-1788	0	*28*	*45*	12	18	—	—	—	6
	Con A	—	10	15	8	12	12	15	—	—

Range of error for each data point shown is consistently less than 15%.

[a] LT activity present in mixed lymphocyte culture (MLC) supernatants was obtained by mixing responder lymphocytes with mitomycin-C treated stimulator cells at a 10:1 ratio for 5 to 6 days at 37°C. LT activity present in antigen or lectin stimulated supernatants was induced by culturing immune lymphocytes with specific antigen (SKSD) at 10 μg/ml or the lectin, concanavalin A (Con A) at 5 μg/ml for 6 days at 37°C.

[b] Peripheral blood mononuclear cells (PBL) obtained after gradient sedimentation on Ficol–Hypaque.

[c] Percent neutralization of LT activity after absorption of 2 ml of supernatant on various numbers of target cells for 30 to 60 min at 4°C with occasional mixing.

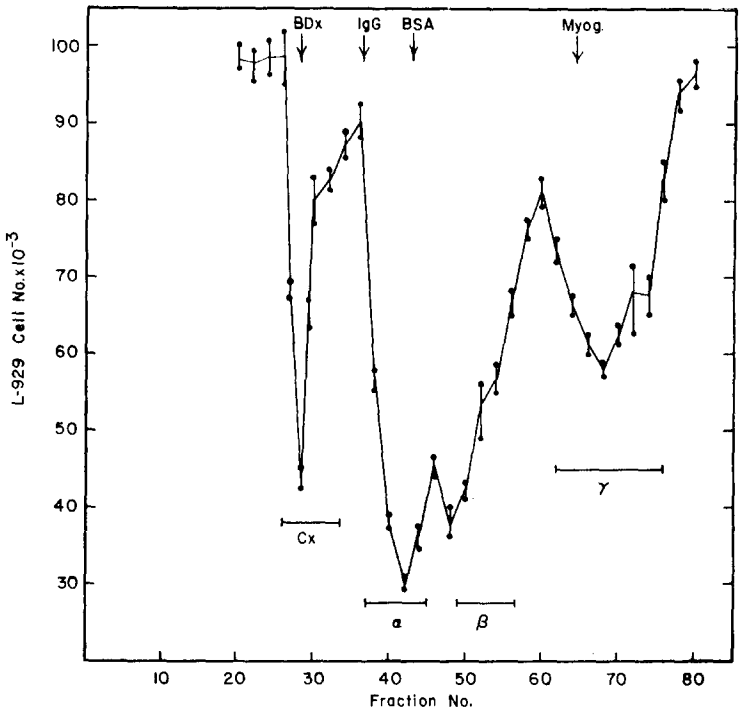

FIG. 3. Elution profile of LT activity after gel filtration chromatography of antigen (SKSD) induced immune human lymphocyte supernatants on Ultrogel AcA 44. Peripheral blood mononuclear cells were obtained and stimulated with 10 μg/ml (200 units/ml) SKSD for 6 days at 37°C. The supernatants were collected, concentrated 30 times, and 2 ml chromatographed on a 2.5 × 100 cm Ultrogel AcA 44 column. Fractions were collected, and 100 μl tested for LT activity on L-929 cells *in vitro*. The elution profile of the various molecular weight markers; Blue Dextran, IgG, BSA, and Myoglobin are indicated by arrows. Fractions collected for pools of the individual LT classes, Cx, α, β, γ are indicated by horizontal lines.

30 times by ultrafiltration, using a PM10 membrane, and 2 ml were chromatographed on a 2.5 × 100 cm Ultrogel AcA 44 gel filtration column. Fractions were collected and 100 μl tested for LT activity on L-929 cells, and the cytotoxic elution profile is shown in Fig. 3. This profile is typical of those reported previously for LT activity in lectin induced lymphocyte supernatants (8, 10). Four major molecular weight classes of LT activity were observed: Complex (> 200,000 D), α (70–90,000 D), β (35–50,000 D), and γ (10–25,000 D). Each of these various LT classes was then pooled (shown by horizontal lines), concentrated by ultrafiltration, and tested for their neutralization by incubation with soluble SKSD or control antigens, TT, or BSA. As shown in Fig. 4, LT activity associated with the large MW complex class is dramatically inhibited (∽75%), while the smaller MW α, β, and γ LT classes are minimally affected (20, 15, and 6%, respectively). In addition, several studies shown in Table 5 reveal that the control protein, BSA, had no demonstrable inhibitory effect on any of the various MW LT classes. This observation is also supported by the finding that the only MW LT class which reacts with anti-Fab′$_2$ serum is the large MW LT complex (Table 5). The data shown in Table 5 also suggests that LT complexes having reactivity with SKSD antigens are identical to those reacting with anti-Fab′$_2$ sera, because incubation of the SKSD

induced LT complexes with both SKSD and anti-Fab'$_2$ sera yields the same level of neutralization as incubation with SKSD or anti-Fab'$_2$ alone. These data strongly support the concept that antigen binding capacity and association with Ig-like receptors are properties unique to the LT complex class.

6. *Reactivity of Human LT Complex with Various Goat Anti-Human Ig Heavy Chain Specific Antisera in Vitro*

To further investigate the molecular nature of the Ig-like molecule(s) present in the LT complex, specific goat anti-human heavy chain antisera were obtained from a commercial source and tested for their neutralizing activity on the complex. As can be seen in Table 6, anti-heavy chain sera with specificities for γ, μ, or δ heavy chain determinants had no apparent inhibitory effect on human LT complex. However, anti-Fab'$_2$ sera was inhibitory, as previously noted. These various antisera were also ineffective when tested against the smaller MW α, β, and γ LT classes on L-929 target cells *in vitro* (data not shown).

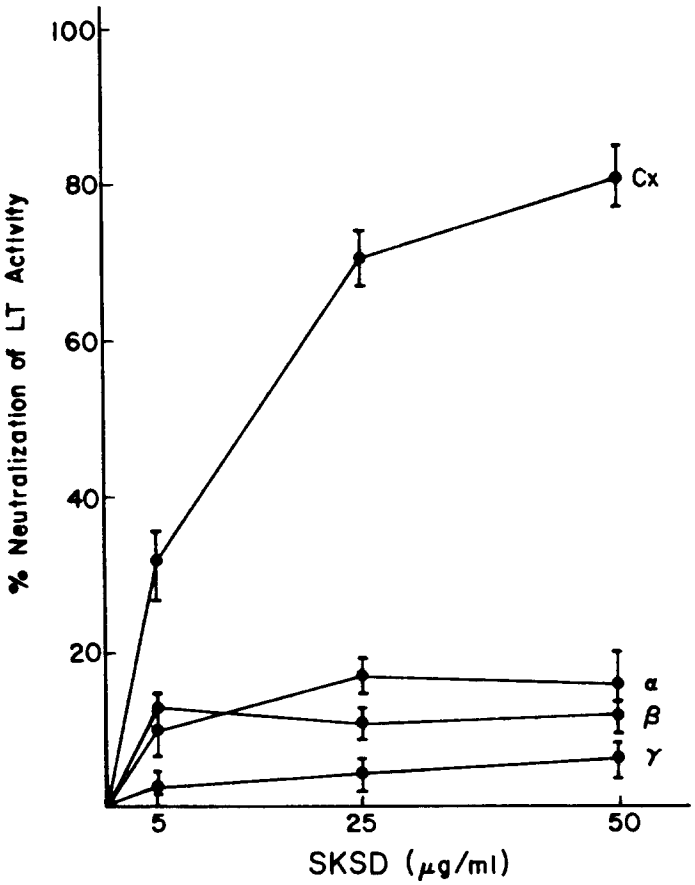

FIG. 4. Inhibition of various LT classes obtained from antigen stimulated immune human lymphocytes by incubation with soluble antigens. The various classes of LT activity, Cx, α, β, γ were obtained after pooling the fractions shown in Fig. 3, concentrating each by ultrafiltration using a PM10 membrane, and then testing for inhibition by soluble antigen as described in Methods.

TABLE 5

Effect of Soluble SKSD and Anti-FAB'$_2$ Sera on LT Activity Present in SKSD Stimulated Human Lymphocyte Supernatants or Separated LT MW Classes

LT class	% neutralization of LT activity by				
	NRS (100 μl)	Anti-Fab'$_2$ (100 μl)	SKSD (50 μg)	BSA (50 μg)	SKSD + Anti-Fab'$_2$ (50 μg + 100 μl)
Whole supernatant	0	27 ± 4	28 ± 4	1	26 ± 6
Complex (Cx-LT)	9	*61 ± 5*	*66 ± 7*	0	*60 ± 5*
α-LT	2	9 ± 3	15 ± 4	0	11 ± 3
β-LT	3	11 ± 4	13 ± 2	2	9 ± 4
γ-LT	0	4 ± 2	2 + 2	0	3 ± 2

Human immune peripheral blood mononuclear cells were obtained and stimulated with 10 μg/ml SKSD for 5 to 7 days at 37°C. The supernatants were then collected, concentrated 30 times and fractionated by gel filtration on Ultrogel AcA 44. The various classes of LT activity obtained from the column profile shown in Fig. 3, or the whole unfractionated supernatant were then tested for their reactivity with SKSD, anti-Fab'$_2$ sera, or both, as described in Methods. LT activity was tested on L-929 target cells and percent neutralization was determined as described in Methods.

DISCUSSION

The present studies, employing heterologous rabbit anti-human Fab'$_2$ sera, suggest that human LT molecules can associate with Ig or "Ig-like" antigen binding receptor molecule(s). This concept was first suggested when we observed that a small percentage of LT activity present in *lectin* (Con A, PHA) activated human lymphocyte supernatants was inhibitable by rabbit antisera directed against the Fab'$_2$ region of normal human IgG molecules (15). The present studies reveal that the inhibition of LT activity by anti-Fab'$_2$ sera was mediated by Ag binding sites, because the neutralizing activity of this antisera was removed by prior incubation with human IgG or Fab'$_2$ fragments. These previous studies on LT molecules released by lectin stimulated cells have been extended in the present studies to

TABLE 6

Inhibitory Effects of Various Heterologous Anti-Human Ig Antisera on Human LT Complex Activity *in Vitro*

Antiserum employed	% neutralization of LT complex lytic activity		
	5 μl	25 μl	100 μl
NRS	—	—	6 ± 1
NGS	0	2	3
Anti-Fab'$_2$	8 ± 2	28 ± 2	49 ± 5
Anti-γ	0	2	0
Anti-δ	1	1	0
Anti-γ, μ, δ	0	1	1

To 200 μl of human LT complex was added various amounts of the above indicated antisera, normal rabbit serum (NRS), or normal goat serum (NGS), and the mixture allowed to preincubate for 1 hr at 4°C. Various amounts of this mixture were then assayed for toxic activity on L-929 cells for 25 hr at 37°C, as described in Methods, and the percent neutralization of toxic activity is expressed relative to untreated controls.

include LT activity released by soluble and cellular antigen stimulated immune cells. We found that while neutralization of toxic activity by anti-Fab'_2 serum was low ($\sim$ 5–20%) in lectin stimulated cultures, it was considerably higher (20–50%) in supernatants obtained from antigen stimulated immune human lymphocytes *in vitro*. We have also shown that the observed toxic activity in these supernatants which is inhibitable by anti-Fab'_2 sera is due to LT molecules, for several reasons: Toxic activity in these supernatants is virtually totally inhibited ($> 95\%$) by goat anti-WS *and* anti-α_2-LT antisera, both potent inhibitors of soluble LT activity *in vitro*. Since the anti-α_2-LT sera employed in these studies does not react with human serum proteins or intact IgG molecules (detected by double diffusion analysis or by immunoabsorption on human serum affinity beads), it seems highly unlikely that inhibition of toxic activity by this anti-LT serum is due to neutralization of "toxic antibody" present in these supernatants. Furthermore, direct testing of Ab molecules present in the serum of a highly sensitive SKSD and TT immune donor reveals very low levels of toxic activity on L-929 cells at Ab levels, which are up to 5000 times the levels present in these culture supernatants. In addition, previous studies have demonstrated that Ab present in normal human, rabbit, and goat sera are not toxic to L cells. Finally, extensive biochemical studies on human LT molecules reveal these materials have physical properties which are very different from classical Ab. Furthermore, complexes of LT molecules, in association with antigen binding receptors can be physically dissociated from the receptor under conditions of high ionic strength (i.e., 0.5 *M* NaCl). These data taken collectively strongly support the concept that cytotoxic LT molecules can associate with nontoxic Ig receptors to form large MW toxic complexes.

The present studies demonstrate that LT molecules obtained from Ag stimulated immune lymphoid cells were, in fact, associated with specific antigen binding receptors. This concept was supported by the findings that these molecules could: 1) bind to and be eluted from affinity columns to which specific soluble antigens were covalently attached; and 2) bind to stimulator cells which induced primary one-way MLC reactions *in vitro*. The greater part of binding of LT activity from MLC cultures to stimulator cells *in vitro* appeared to be immunologically specific, for only the identical stimulator cells would absorb significant levels of LT activity. In contrast, absorption of these supernatants with nonrelated target cells removed only low levels of LT activity. In addition, LT activity in the supernatant from lectin or soluble antigen stimulated lymphoid cells was not removed by absorption with these same stimulator cells. Furthermore, these absorptions were carefully performed at 4°C, to eliminate loss of LT activity by nonspecific means such as cell surface protease digestion or pinocytosis. These studies strongly support the concept that immune lymphoid cells stimulated with a specific (soluble or cellular) Ag can release LT molecules which are physically associated with specific Ag binding receptor(s).

It appears that the antigen binding receptor(s) detected in these supernatants are only in association with the complex high MW forms of LT. Previous physical and immunological studies on LT molecules obtained from lectin stimulated human lymphocytes *in vitro* have demonstrated that these molecules represent a related system of cell toxins which can interact together in various combinations to form large MW complexes (14, 15). Furthermore, this assemblage of LT molecules in the complex form appears to be at least partially noncovalent for lytically active,

smaller MW LT components (α, β) can be removed from the complex class by treatment with high ionic strength buffers (15). In these same studies, we found that the smaller MW LT classes appeared to require a nontoxic condensing molecule to form the complex which was distinct from Ag binding receptor. While these previous studies documented the observation that cell lytic activity associated with human LT complex could be inhibited by incubation with rabbit anti-human Fab'_2 sera, they only suggested that the LT complex may have antigen binding capacity if derived from immune lymphoid cells. The present studies reveal that LT activity present in supernatants obtained from soluble antigen or MLC activated immune human lymphoid cells *in vitro* does, indeed, possess Ag binding capacity. Furthermore, it became clear after fractionation of supernatants obtained from soluble Ag stimulated immune cells into the various MW LT classes that both Ag binding capacity and reactivity with rabbit anti-human Fab'_2 sera were functions of the large MW LT complex class. The smaller MW α, β, and γ LT classes neither react with anti-Fab'_2 sera nor exhibit significant levels of Ag binding activity. Thus, it appears that while the smaller MW human LT classes do not, in themselves have antigen binding receptor activity, these molecules can coalesce together in various combinations to form large complexes. These complexes may then form the receptor themselves or further associate with Ag binding receptor molecules. It is noteworthy that there appears to be a functional heterogeneity among the various LT complexes. We found in a given supernatant or sephadex fraction, LT complexes which are associated with Ig molecules and others which are not. Indeed, only a portion of the complex class is inhibitable by anti-Fab'_2 sera or will bind to specific antigen columns. Furthermore, physical heterogeneity of LT complexes isolated from lectin stimulated human lymphocytes *in vitro* has been noted. These molecules can be resolved into multiple but discrete and perhaps interconvertible MW forms ranging in size from 200,000 to 10^6 D. Preliminary findings suggest that only certain forms are associated with Ag binding receptors, and moreover, some forms are highly unstable.

A previous study has noted antigenic relationships between human LT activity released *in vitro* from certain continuous human lymphoid cell lines and human Ig molecules (23). The nature of the "Ig-like" materials we have identified in these LT complexes, or the lymphoid cell type from which they come, are as yet unknown. Studies reported in this manuscript, employing *goat* anti-human heavy chain specific antisera suggest classical Ig heavy chain determinants are not detectable in the LT complex. However, there are at least three interpretations of these findings: 1) heavy chain determinants are expressed and these antisera do recognize them, but that interaction does not inhibit LT complex lytic activity detectable on L929 cells *in vitro*; 2) heavy chain determinants present in these complexes may be different from the classical μ, γ, or δ heavy chains present in serum Ig; or 3) classical Ig heavy chains are present but are masked by surrounding LT molecules. This latter concept is similar to the finding that certain complement components become antigenically "masked" when they assemble into the terminal C'5-9 lytic complex (24). It is clear, however, that the reactivity of these LT complexes with anti-Fab'_2 sera, as well as their capacity to bind to specific antigen immunoabsorbent columns strongly suggests the presence of Ig-like structures. It is noteworthy that on occasion, we have seen significant neutralization of complex or α-LT activity by commercially available *rabbit* anti-Ig antisera (i.e., anti-IgG,

or anti-μ or γ heavy chain specific sera). Thus, it is possible that recognition of the Ig-like determinants present in these LT-Ig associated complexes may depend upon the species of animal used to develop the appropriate antisera. This finding may, in fact, be relevant to a similar situation in which murine T cells are not stained by rabbit or goat anti-mouse Ig sera (25), but are stained by chicken anti-mouse Ig sera (26). It is also conceivable that neutralization of LT complex activity by anti-Fab'$_2$ sera is due to reactivity of this sera with light chain determinants.

The class of lymphoid cells involved in the synthesis and/or release of various LT molecules and Ig-like receptors are also under current study. Clearly, this situation is complex, but there is evidence that indicates different lymphoid cells can release various forms of LT. The identity of the cell(s) involved in synthesis and release of the Ig-like receptor associated with these complexes is unknown. However, identification of the type of receptor present in these complexes will help elucidate the class of lymphocyte from which it originates. It is not clear whether the same cell releases both the Ag binding receptor molecules and the LT molecules together in the complex forms, or whether these molecules associate after release. These interesting questions will require further study.

The finding that cell-lytic LT molecules can associate with antigen binding receptors reveals an important new concept, for it provides a means by which cytolytic molecules could become highly specific when directed through the Ig receptor molecule. We feel it is significant that the antigen binding receptor is only associated with the high MW complex form(s) of LT, and that these represent biologically relevant macromolecules for the following reasons: 1) the finding that the smaller MW forms of LT interact together to form a high MW complex implies that this is a related system of molecules which may not act as individuals but function collectively in a fashion perhaps analogous to the complement system; 2) that these LT complexes when directed through the Ag binding Ig-like receptor(s) may, in fact, be more lytically effective than the smaller MW classes. Extensive studies in preparation for the next manuscript in this series strongly support this concept.

It is clear LT molecules do participate as lytic effectors in certain forms of lymphocyte mediated cytotoxicity *in vitro*, for several studies have shown that anti-LT sera can inhibit certain forms of these reactions (6, 7). However, the mechanism by which anti-LT sera inhibit cytotoxicity will require extensive study. Our understanding of the mechanism(s) involved in lymphocyte mediated cell lysis *in vitro* will require that we further investigate the biochemical nature of these LT molecules, the assemblage of these molecules into large MW complexes, their expression on the effector lymphocyte plasma membrane, and how these complexes become associated with Ig-like receptor(s).

ACKNOWLEDGMENTS

The authors wish to thank St. Josephs Hospital, Orange, California, for providing tissue samples, Irene Shimizu for donating blood, Gloria Stangl for preparation of this manuscript, and LT Furbrush for excellent technical assistance.

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