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The Human LT System[1]

III. Characterization of a High Molecular Weight LT Class (Complex) Composed of the Various Smaller MW LT Classes and Subclasses in Association with Ig-Like Molecules

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Cytotoxic activity (lymphotoxin (LT)) present in supernatants from lectin stimulated human lymphocytes *in vitro* is composed of a heterogeneous system of biological macromolecules which can be separated into multiple classes and subclasses on the basis of their molecular weight and charge. These studies further characterize a large molecular weight human LT class, termed complex (MW >200,000 d), which elutes in the void volume off Sephadex G-150 or Ultrogel AcA 44. Immunological studies on the complex, employing various rabbit anti-LT class and subclass antisera, revealed this material is a macromolecular assemblage of the smaller MW α, β, γ LT classes and subclasses. Furthermore, the reactivity of this material with anti-human Fab'_2 (IgG) indicates these smaller molecular weight LT components can associate with immunoglobulin or Ig-like molecules. The materials present in the LT complex class appear to be noncovalently associated, since conditions of high ionic strength dissociate certain small MW LT components, while low ionic strength buffers may cause these components to reaggregate with the complex. When subjected to velocity sedimentation on sucrose gradients or gel filtration on Ultrogel AcA 22, LT complex activity elutes as several discrete peaks of activity in the 200,000 to 1,000,000 MW range. These findings suggest the concept that LT molecules can form discrete and specific macromolecular structures which contain the smaller MW LT classes. Moreover, these structures can also associate with immunoglobulin-like molecules to form secondary LT-Ig complexes. This may have important biological significance in explaining how nonspecific cell toxins could play a role in specific or nonspecific cell lytic reactions *in vitro*.

INTRODUCTION

Physical-chemical studies of human LT molecules indicate they are heterogeneous and can be physically separated and functionally purified one from another into several classes and subclasses based on their molecular weight and charge (1–4). Immunologic studies of these molecules has indicated that the various classes and subclasses bear common and distinct antigenic determinants, which support the concept that they represent an interrelated group of molecules which may be able to as-

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sociate with one another into various MW forms. During physical-chemical studies, a large molecular weight class, termed complex, of greater than 200,000 daltons, was identified. Immunologic studies suggested this material(s) contained the smaller molecular weight LT classes.

This manuscript deals with further physical-chemical and immunologic studies on the nature of the complex class of LT activity found in the supernatants of PHA activated human lymphocyte cultures. These data support the concept that LT molecules can form discrete and specific macromolecular structures which contain the smaller MW classes. Moreover, these complexes may further interact with other macromolecules, one of which is immunoglobulin-like, to form secondary LT-Ig associations which may have important biological significance in explaining how nonspecific cell toxins could play a role in specific cell lytic reactions *in vitro*.

MATERIALS AND METHODS

I. Tissue Culture Techniques and Cytotoxic Assays

The methods employed for maintaining cell lines of L-929 fibroblasts or normal human lymphocyte cultures *in vitro* has been previously described in detail in the first manuscript of this series (1). Methods for production of human lymphocyte supernatants and testing for the presence of LT activity are also described in the same manuscript.

II. Gel Filtration Chromatography

The methods employed and columns used for gel filtration chromatography (Ultrogel AcA 44 or AcA 22) were previously described in the first manuscript of this series (1).

III. Production and Use of Anti-LT antisera

The details for physical separation of the various LT classes and subclasses used for immunization and testing, immunization protocols, collection of antisera, and testing of antisera have been thoroughly described in the first and second manuscripts of this series and elsewhere (1, 5, 6).

IV. Preparation of Rabbit Anti-human Fab'$_2$ IgG Affinity Column

Anti-human Fab'$_2$ antisera was obtained after repeated immunizations of rabbits with human Fab'$_2$ suspended in CFA. Fab'$_2$ fragments were obtained after chromatography on Spehadex G-150 of pepsin treated human IgG, which was purified from pooled normal human serum by 50% $(NH_4)_2SO_4$ precipitation and chromatography on DEAE-cellulose, as described by Williams and Chase (7). The IgG fraction of this anti-Fab'$_2$ antisera was then further purified by passage over a Sepharose 4B affinity column to which human IgG was covalently linked (8) and eluted with two column volumes of 0.1 *M* acetate buffer containing 0.5 *M* NaCl, pH 2.4. After elution, the fractions containing IgG were neutralized by addition of 1 *M* NaOH. Extensive physical-chemical, and immunologic studies verified the eluted material was IgG, and that this IgG only reacted with intact human immunoglobulin molecules. Double diffusion analysis of this anti-Fab'$_2$ sera against

whole human serum, human IgG, and Fab'$_2$ obtained from IgG revealed single precipitation bands with no spurs, indicating reactions of identity. Anti-human Fab'$_2$ IgG was then coupled to CNBr activated Ultrogel AcA 44 beads by the method of March and Cuatrecassas (8), and equilibrated in PBS before use. Two mg of IgG was bound/ml beads. Four milliliters of beads were packed into a 6 ml plastic syringe, and 1 to 2 ml of rechromatographed complex LT was applied and allowed to interact with the beads for 30′ at 25°C. The column was then washed with four column volumes of PBS followed by 4 ml of 2 *M* KI, pH 9.0, in 0.01 *M* phosphate. Fractions containing the KI were then dialyzed overnight against 1000 volumes of PBS at 4°C. One-hundred microliters was then tested for toxic activity as previously described.

V. Velocity Sedimentation on Sucrose Gradients

Molecular weight determinations by velocity sedimentation on sucrose gradients were performed in the following manner. A 12 ml linear sucrose gradient (5–20% sucrose) in 0.02 *M* phosphate, pH 7.2, and various concentrations of NaCl, was formed in 15 ml polyallomer centrifuge tubes. To each individual gradient, 100 μl of a sample containing 1 mg each of various protein markers or 50 to 100 units of LT activity were applied. The gradients were then centrifuged at 38,000 rpm for 8 to 24 hr on a Beckman Model L65 ultracentrifuge. The gradients were then punctured at the bottom and 0.30 ml fractions collected. The position of the various marker proteins was determined by absorbence at 280 nm, while cytotoxic activity was analyzed identical to the method used for the gel filtration columns already described (1).

VI. Stability to Heat, Salt, and Treatment with DNAse or Trypsin

One mg (1000 units of DNAse I (Sigma, St. Louis, Mo.) was suspended in 1 ml, 0.01 *M* phosphate, pH 7.2. Fifty microliters of this solution (50 units DNase) was then added to 200 μl of LT complex or control buffer in 0.01 *M* phosphate, pH 7.2, containing 10 m*M* $MgCl_2$ and 10 m*M* $CaCl_2$, and allowed to incubate for 2 to 4 hr at 37°C. After this time, 20 μl of 10^{-2} *M* EDTA was added to stop the reaction and remaining toxic activity was determined. To test the effect of trypsin on complex LT activity, 50 μl of a 0.5% trypsin (crystallin trypsin, Difco) solution in PBS containing 10^{-4} *M* EDTA was added to 200 μl of LT complex or control buffer, in 0.01 *M* phosphate, pH 7.2, 10^{-4} *M* EDTA, and allowed to incubate for 2 or 4 hr at 37°C. Toxic activity was then tested by adding 20 μl to duplicate 1 ml tube cultures of L-929 target cells in media containing 3% fetal calf serum to inhibit further trypsin activity.

RESULTS

I. Resolution of LT Complex from α and β-LT Activity by Gel Filtration Chromatography

Cytotoxic activity present in supernatants of lectin stimulated human tonsil and adenoid lymphocytes *in vitro* (SAL) can be physically separated into several molecular weight classes by gel filtration chromatography on Ultrogel AcA 44 or Sephadex G-150. Shown in Fig. 1A is the elution profile of 2 ml of a 5 day 50 ×

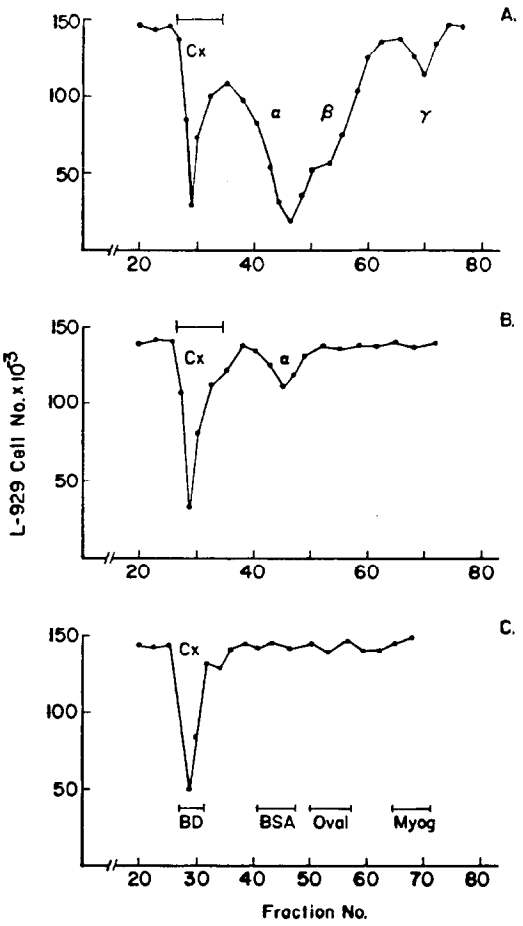

FIG. 1. Resolution of LT complex class from α, β, and γ-LT classes by chromatography on Ultrogel AcA 44. A. Elution profile of LT activity present in 2 ml of a 5 day 50 × concentrated SAL on Ultrogel AcA 44, equilibrated in 0.01 *M* phosphate, pH 7.2, 10^{-4} *M* EDTA. Fractions were collected, and 0.10 ml tested in duplicate for toxic activity on L-929 target cells. Standard error in duplicate cell counts is routinely less than 10%. Fractions collected for rechromatography (Fraction 26–34) are indicated by a horizontal line. B. The fractions collected in A were concentrated to 2 ml on a PM10 membrane and rechromatographed on the same Ultrogel AcA 44 column. Fractions were collected, and 0.10 ml tested in duplicate for toxic activity. Fractions collected for rechromatography (Fractions 26–34) are indicated by a horizontal line. C. Fractions collected in B were concentrated to 2 ml on a PM10 membrane and rechromatographed on the same Ultrogel AcA 44 column. 0.10 ml was tested for toxic activity. The position of the various molecular weight markers, Blue Dextran (BD), bovine serum ablumin (BSA), ovalbumin (Oval), and Myoglobin (Myo) are indicated by horizontal lines.

concentrated SAL on Ultrogel AcA 44 equilibrated in 0.01 *M* phosphate, pH 7.2, 10^{-4} *M* EDTA. Clearly, α, β, and γ LT classes are present as described previously (1). In addition, a large MW component eluting in the void volume, which we term complex, is also resolved by this procedure. The percentage of total activity present in the LT complex peak is variable but ranges from 5 to 20% of the total LT activity in a given supernatant. When fractions containing LT complex activities are pooled (fractions 26–34 pooled—shown by horizontal bar), concen-

trated, and rechromatographed on the same Ultrogel AcA 44 column, multiple peaks of toxic activity are again observed with molecular weights corresponding to LT complex and α-LT (Fig. 1B). It is not yet clear whether this peak of α-LT activity is due to contaminating α-LT molecules in the fractions collected for the LT complex pool, or are breakdown products from the LT complex during preparation for rechromatography. However, when fractions containing LT complex activity shown in Fig. 1B are again pooled (fractions 26–34), concentrated, and rechromatographed, only LT complex activity is observed (Fig. 1C). These rechromatography experiments have been performed 6 to 8 times employing both salt-free and physiologic saline buffers yielding similar results. However, a general observation is that by employing salt-free buffers, aggregation of the smaller MW LT molecules in the presence of the complex may occur, thereby giving larger peaks of LT complex activity.

II. Evidence that LT Complex Activity is Not a "Nonspecific Aggregate"

Several different experiments were performed to eliminate the possibilities that LT complex activity was due to nonspecific aggregation of smaller LT classes with themselves or other unrelated proteins during concentration and handling of SAL. These experiments revealed: (1) LT complex activity is detected in unconcentrated SAL, eliminating the possibility that the activity is a concentration artifact; (2) LT complex activity is present in SAL which contains serum, serum substitutes, or which is *serum free*. This reduces the possibility that LT complex results from smaller LT classes or subclasses "riding" on *exogenous* large MW serum protein; (3) to eliminate the possibility that LT complex was formed as a result of simple affinity of LT molecules for themselves in culture, the following experiment was performed: Two milliliters of a 5 day 50 × concentrated SAL was applied to Ultrogel AcA 44 in PBS, and the elution profile is shown in Fig. 2A. All fractions except those containing LT complex activity (fractions 36—phenol red) were then pooled, concentrated to the original 2 ml, and reapplied to the column, and separated in 0.01 *M* phosphate, pH 7.2 and no salt. Clearly, Fig. 2B shows that this process does not facilitate the formation of new LT complex activity, yet α and β-LT classes are essentially unaltered. This experiment has been repeated three times employing both salt-free and salt containing (PBS) buffers yielding identical results.

III. Molecular Weight Determination of LT Complex by Velocity Sedimentation on Sucrose Gradients or Molecular Sieving on Ultrogel AcA 22

A. Sucrose gradients. Sucrose gradients were formed with a 5 to 20% linear sucrose solution in 0.01 *M* phosphate buffer, pH 7.2, 10^{-4} *M* EDTA, and varying concentrations of NaCl. Each gradient received 100 μl of rechromatographed LT complex (containing 20–80 units of cytotoxic activity) or 100 μl of buffer containing 1 mg each of bovine thyroglobulin (MW 660,000) and human IgG (MW 150,000) as reference MW markers. The gradients were then centrifuged at 38,000 rpm for 8 to 10 hr, after which 0.30 ml fractions were collected from the bottom of the centrifuge tube and 100 μl tested for cytotoxic activity, as described in *Methods*. The positions of the molecular weight markers were determined by absorption at 280 nm. Figure 3 shows representative results of eight different experiments. Clearly, LT complex activity has a heterogeneous distribution of

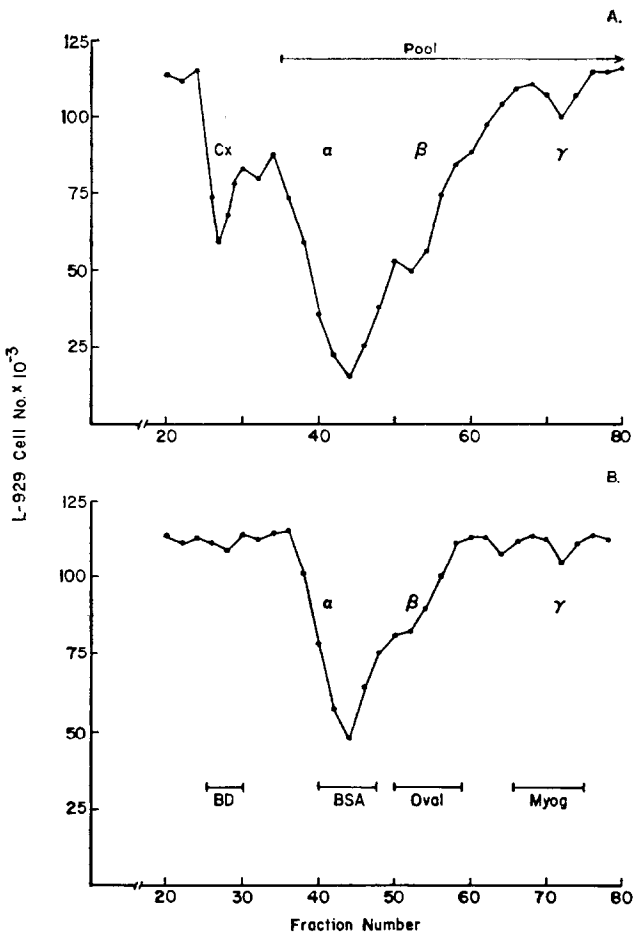

FIG. 2. Capacity of α, β, and γ-LT classes to form complex when concentrated together *in vitro*. A. Elution profiles of LT activity present in 2 ml of a 5 day 50 × concentrated SAL on Ultrogel AcA 44, equilibrated in PBS. The fractions containing α, β and γ class LT activities (Fractions 36–80, indicated by horizontal arrow) were collected and concentrated to the same 2 ml volume in which they were originally present. The sample was then applied to the same Ultrogel column but equilibrated in 0.01 *M* phosphate, pH 7.4, 10^{-4} *M* EDTA. B. Elution profile of the concentrated α, β, and γ-LT classes obtained as described in A.

cytotoxic activity on sucrose gradients. Under salt-free conditions (Fig. 3A), the complex appears to sediment in four basic MW ranges (1) 800,000 d; (2) 400 to 600,000 d; (3) 200 to 300,000 d; and (4) ∽150,000. However, under conditions where the ionic strength of the buffer is increased to 0.15 *M* NaCl (PBS), the amounts of activity associated with the higher MW forms are not as evident (Fig. 3B). Moreover, under conditions of higher ionic strength buffers (0.5 *M* NaCl), the major peak of toxic activity elutes just behind the IgG marker.

B. Molecular Sieving. An aliquot (2 ml) of the same sample of LT complex which was applied to the sucrose gradients was also applied to a 2.5 × 100 cm column containing Ultrogel AcA 22, equilibrated in 0.01 *M* phosphate, pH 7.2, 10^{-4} *M* EDTA. Fractions were collected, and 100 μl was tested for toxic activity as described in *Methods*. As can be seen in Fig. 4, LT complex is also heterogeneous

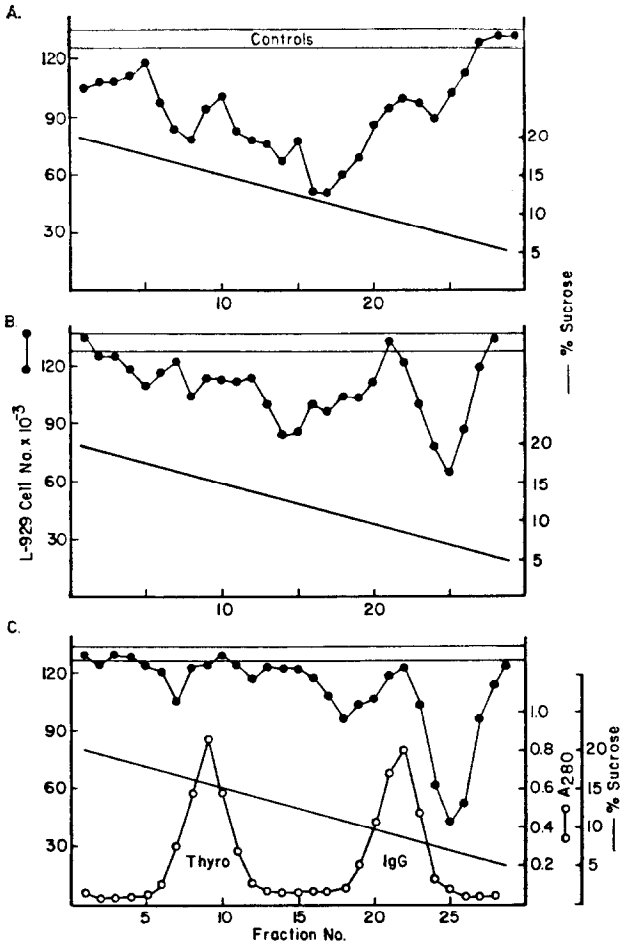

FIG. 3. Velocity sedimentation of LT complex class on linear sucrose gradients with various concentrations of NaCl. A. One-hundred microliters of rechromatographed LT complex was applied to a 12 ml, 5 to 20% linear sucrose gradient in 0.01 *M* phosphate pH 7.2, 10^{-4} *M* EDTA. After 8 hr of centrifugation at 38,000 rpm, 0.3 ml fractions were collected from the bottom of the tube, and 0.1 ml tested for toxic activity. B. Same as in A, except sucrose gradient contained PBS as the buffer. C. Same as in A, except sucrose gradient contained 0.01 *M* phosphate, pH 7.4, 10^{-4} *M* EDTA, and 0.5 *M* NaCl as the buffer. The position of the various molecular weight markers (Thyroglobulin and IgG) is also shown. These markers were applied to a separate gradient but centrifuged simultaneously with the LT complex gradients. The position of the reference MW markers was not altered by varying the ionic strength.

when fractionated by this procedure. However, four major distinct peaks of activity are observed, having approximate MW of: (1) ~1,000,000 d; (2) 600 to 800,000 d; (3) 400 to 600,000 d; and (4) 100 to 200,000 d; as determined by the reference MW markers. These peaks have been termed Cx1, Cx2, Cx3, and Cx4, respectively. To support the concept that these various peaks represent discreet molecular forms of complex, each peak was further rechromatographed on Ultrogel. Fractions containing Cx1, Cx2, Cx3, and Cx4 LT activity were collected, concentrated, and again rechromatographed on Ultrogel AcA 22. Clearly, Fig. 4b, c, d, and e show that these peaks elute with identical profiles, as observed in the original

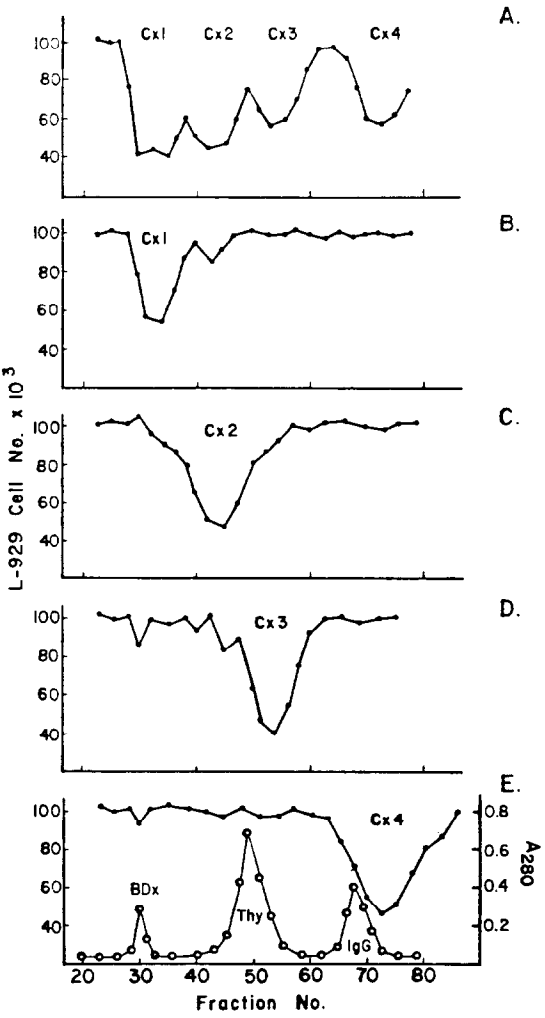

FIG. 4. Resolution of multiple molecular weight forms of LT complex class by gel filtration on Ultrogel AcA 22. A. Elution profile of toxic activity present in the LT complex class on Ultrogel AcA 22. Two milliliters of rechromatographed LT complex was applied to Ultrogel AcA 22, equilibrated in 0.01 *M* phosphate, pH 7.2, 10^{-4} *M* EDTA. Fractions were collected and 0.10 ml tested for toxic activity. Fractions containing individual peaks of LT complex activity, indicated as Cxl through Cx4, were collected, concentrated on a PM30 membrane to 2 ml, and rechromatographed on the same Ultrogel AcA 22 column. B. Rechromatography of Cxl on Ultrogel AcA 22. C. Rechromatography of Cx2 on Ultrogel AcA 22. D. Rechromatography of Cx3 on Ultrogel AcA 22. E. Rechromatography of Cx4 on AcA 22, equilibrated in the same buffer as described above. The position of the various MW markers are indicated by absorbence at 280 nm.

Ultrogel AcA 22 separation. Additional studies on Cx4 have revealed that this material also contained α-LT activity. These experiments have been performed several times, employing both salt-free and salt containing (PBS) buffers, yielding identical results.

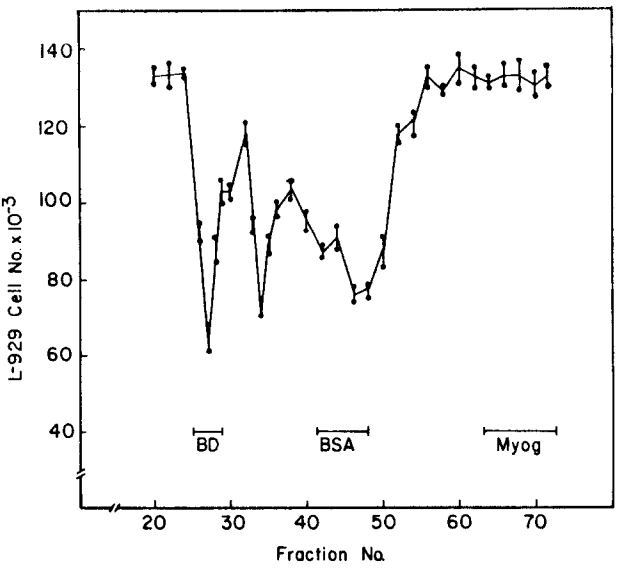

FIG. 5. Gel filtration chromatography of LT complex on Ultrogel AcA 44 equilibrated in 0.5 *M* NaCl. Rechromatographed LT complex was obtained under conditions of 0.01 *M* phosphate, 10^{-4} *M* EDTA, pH 7.2. One-half of the sample (2 ml) was dialyzed overnight at 4°C, against 1000 V of 0.5 *M* NaCl in 0.01 *M* phosphate, pH 7.4, and rechromatographed on Ultrogel AcA 44, equilibrated in 0.5 *M* NaCl. Fractions were collected, and 0.10 ml tested in duplicate for toxic activity. The elution profile of the sample left untreated is shown in Fig. 1C.

IV. Disassociation of Small MW LT Components from the LT Complex Class by Treatment with 0.5 M NaCl

To test the concept that LT complex is a noncovalent assemblage of smaller MW LT components, fractions containing rechromatographed LT complex were obtained and adjusted to 0.5 ml NaCl, or left unadjusted. The sample adjusted to 0.5 *M* NaCl was then chromatographed on Ultrogel AcA 44 preequilibrated in 0.01 *M* phosphate, 10^{-4} *M* EDTA, and 0.5 *M* NaCl. The untreated sample was also applied to Ultrogel AcA 44 in 0.01 *M* phosphate and no salt. A representative result of two such experiments is shown in Fig. 5. As can be seen, LT complex activity appears to elute in at least three resolvable peaks under conditions of 0.5 *M* NaCl. Peaks 1 and 3 correspond to LT complex and α-LT activities, respectively. However, a new peak is observed in the 140 to 160,000 MW range. These identical results have been obtained in two similar experiments. Figure 1C shows the results when LT complex is rechromatographed on Ultrogel AcA 44 in no salt. Clearly, no smaller MW LT forms are observed.

V. The Effect of Various Physical and Enzymatic Treatment on Complex LT Activity In Vitro

The next experiments were designed to test the effects of various physical (heat, salt) or enzyme treatments (trypsin, DNAse) on complex LT activity *in vitro*. To test the effects of heat or salt, 200 μl of LT complex, containing approximately 20 units of toxic activity, was heated at 56° or 85°C or adjusted to 1 *M* NaCl by adding 50 μl of 5 *M* NaCl, and incubating at 37°C for 2 or 4 hr. The remaining

TABLE 1

Stability of Human LT Complex to Various Physical and Enzymatic Treatments *in Vitro**

Treatment	% Control LT complex activity	
	2 hr	4 hr
Untreated	100	100
56°C	100	90
85°C	5	2
1 *M* NaCl	100	100
DNAse (50 units)	95	92
Trypsin (0.01%)	25	19

* 200 microliters of rechromatographed complex LT activity (containing approximately 20 units of activity) was tested for its stability at 50 or 85°C, and to 1 *M* NaCl at 37°C, for 2 to 4 hr. Sensitivity to the enzymatic activity of trypsin or DNAse was tested by adding 50 μl of DNAse I to 200 μl of complex LT or 50 μl of 0.5% trypsin to 200 μl LT complex, as described in Methods. After 2 or 4 hr at 37°C, remaining toxic activity was then determined as described in *Methods*.

toxic activity was tested by adding 100 μl to 1 ml cultures of L-929 cells, and incubating over 24 hr at 37°C. Similar fractions of LT complex activity were tested for their susceptibility to treatment with DNAse or trypsin, as described in *Methods*. As can be seen in the data presented in Table 1, LT complex activity is not affected by treatment with 1 *M* NaCl or 56°C, up to 4 hr. However, the activity is totally destroyed when heated to 85°C for 2 hr. In addition, while the activity appears to be resistant to DNAse treatment, 80% of the lytic activity is sensitive to trypsin in 2 hr at 37°C.

VI. Immunological Reactivity of LT Complex with Rabbit Antisera Directed Against Various Classes and Subclasses of Human α and β-LT In Vitro

Experiments were next initiated to determine whether rabbit antisera directed against various classes and subclasses of human α and β-LT could recognize similar determinants present on the LT complex. For these experiments, 25 μl of various rabbit anti-LT sera or normal rabbit serum (NRS), which were described in the previous manuscript (5), were incubated with 1 ml of rechromatographed LT complex containing approximately 25 units of cytotoxic activity in RPMI-S for 1 hr at 37°C. Shown in Table 2 is a compilation of the results obtained from several different experiments. Clearly, antisera directed against different classes and subclasses of human α and β-LT inhibit LT complex cytotoxic activity when tested on L-929 target cells *in vitro*. However, inhibition was variable, depending upon the type of antisera employed. The best inhibition was obtained using anti-class specific antisera, that is, antisera directed against *all* members of the α-LT class of cytotoxins (anti-α-LT). This antisera can inhibit 90 to 100% of LT complex cytotoxic activity. Similar results were obtained with polyspecific antisera which are directed against materials present in a whole supernatant (anti-WS). While anti-complex antisera (anti-C_x) partially neutralized complex lytic activity (60–70%), antisera directed against different subclasses of α-LT (i.e., anti-α_1 or anti-α_2) also only partially inhibited LT complex activity. While anti-α_1 sera neutralized 40 to 60% of LT complex activity, anti-α_2 sera neutralized 65 to 80% of its activity.

Finally, both anti-β_1 and anti-β_2 subclass were neutralized 60 to 100% of LT complex activity.

VII. Immunological Reactivity of LT Complex with Rabbit Antisera Directed Against Human IgG (Fab'$_2$) In Vitro

It has been reported that cytotoxic activity present in supernatants of certain continuous human lymphoid cell lines has reactivity with heterologous antisera directed against the F_c region of human IgG or human γ light chains (9). We decided to test the reactivity of the various LT classes obtained from lectin stimulated normal lymphocytes with heterologous antisera directed against human Fab'$_2$ (IgG). The anti-Fab'$_2$ sera was obtained and characterized as described in *Methods*. Reactivity of this antisera with the various LT classes was performed as follows: To 1 ml of rechromatographed LT complex, α-LT or β-LT activity was added 10 to 200 μl of anti-Fab'$_2$ serum, 50 to 500 μg of its IgG fraction or NRS, and allowed to incubate for 1 hr at 37°C. Various amounts of the samples (10–100 μl) were then placed directly on L-929 target cells and tested for cytotoxic activity. Shown in Table 3 are the representative results of three such experiments. Clearly anti-Fab'$_2$ sera or its IgG fraction can strongly inhibit LT complex activity. Generally, 50 to 70% of the LT complex activity was blocked by 100 μl of antisera. In contrast, anti-Fab'$_2$ sera did not significantly react with fractions containing α or β class LT activity. Furthermore, neither NRS, anti-ovalbumin, nor anti-BSA serum inhibited LT complex, α-LT or β-LT classes. These data suggest that LT complex activity is associated with some form of Ig molecule which is not immunologically detectable in the smaller MW classes.

The reactivity of LT complex with anti-Fab'$_2$ (IgG) was further examined by its capacity to selectively bind to affinity columns to which the IgG fraction of this antisera was covalently bound. In these experiments, 2 ml of rechromatographed LT complex activity in PBS (containing 100–200 units of LT activity) was slowly eluted through an affinity column containing 4 ml of packed Ultrogel beads (2.0 mg IgG/ml beads) at 25°C. After allowing a 30 min binding period at 25°C, the

TABLE 2

Neutralization of LT Complex Activity by Various Rabbit Anti-LT Antisera *in Vitro*

Antisera specificity	% Neutralization of LT complex
Anti-WS	90–100
Anti-Complex	60–70
Anti-α-LT (class)	90–100
Anti-α_1-LT (subclass)	40–60
Anti-α_2-LT (subclass)	65–80
Anti-β_1 or β_2-LT	60–70, 80–100

To 200 μl of rechromatographed LT complex (containing approximately 20 units of LT activity) was added 25 μl of various rabbit anti-LT sera or NRS, and the samples were allowed to incubate at 37°C for 1 hr. After this time, 100 μl of each sample was tested for remaining toxic activity on L-929 cells, as described in *Methods*. Percent neutralization is determined relative to untreated or NRS treated samples by the following formula:

$$\% \text{ Neutralization} = \frac{(\text{Cell No.} + \text{Ab} + \text{LT}) - (\text{Cell No.} + \text{LT} + \text{NRS})}{(\text{Cell No.} + \text{NRS}) - (\text{Cell No.} + \text{LT} + \text{NRS})} \times 100.$$

TABLE 3

Reactivity of Human LT Complex, α, and β-LT Activities with Various Antisera *in Vitro*

Class of LT	Antisera	Amount employed	% Neutralization
LT Complex	Anti-Fab'$_2$	10 μl	17 ± 2
LT Complex	Anti-Fab'$_2$	50 μl	41 ± 5
LT Complex	Anti-Fab'$_2$	100 μl	58 ± 6
LT Complex	Anti-Fab'$_2$	200 μl	60 ± 4
LT Complex	Anti-Fab'$_2$ IgG F_x	500 μg	68 ± 4
LT Complex	Anti-Ovalbumin	100 μl	2 NS
	Anti-BSA	100 μl	5 NS
	NRS	100 μl	3 NS
α-LT	Anti-Fab'$_2$	100 μl	5 NS
	Anti-BSA	100 μl	2 NS
	NRS	100 μl	4 NS
β-LT	Anti-Fab'$_2$	100 μl	11 ± 2
	Anti-BSA	100 μl	3 NS
	NRS	100 μl	0 NS

To 1 ml of rechromatographed LT complex, α-LT, or β-LT activities (containing approximately 50 units/ml) was added various amounts of anti-Fab'$_2$ sera, anti-ovalbumin, anti-BSA, or NRS, and allowed to incubate for 1 hr at 37°C. After this time, remaining toxic activity was tested by adding 50 or 100 μl of the sample to L-929 cells, as described in *Methods*. % neutralization is determined relative to nontreated or NRS treated samples and calculated as in Table 2.

NS = Not significant.

column was washed with 20 ml PBS, followed by 4 ml of 2 *M* KI (in PBS). Fractions containing KI were dialyzed overnight at 4°C, against 1,000 Vol. PBS, and each fraction was then tested for cytotoxic activity by adding 100 μl to 1 ml tube cultures of L-929 cells during a 24 hr incubation at 37°C. Shown in Fig. 6 is a representative result of two such experiments. Clearly, two peaks of toxic activity are observed when LT complex is eluted through an anti-Fab'$_2$ (IgG) affinity column. A nonbinding peak, which represents approximately 30 to 40% of toxic activity and a binding peak representing 60 to 70% of total activity. In contrast, when the same amount of LT complex is passed over similar columns containing Ultrogel beads to which BSA or normal rabbit IgG are coupled, only a single nonbinding peak of activity is observed (data not shown). Thus, the selective binding of LT complex to anti-Fab'$_2$ IgG is a function of Fab determinants present on these molecules.

DISCUSSION

The present manuscript examines some of the physical properties of a high molecular weight form of human LT activity we have termed LT complex. This material is identified as an LT class on the basis of its molecular weight. All complex LT activity elutes in the void volume off Sephadex G-150, or Ultrogel AcA 44, and is thus larger than 200,000 daltons. This activity appears to be due to protein, for it is trypsin sensitive, DNAse resistant, and while stable to heating at 56°C, and to exposure to 1 *M* NaCl, it is destroyed at 85°C. The amount of activity in a given supernatant associated with LT complex varies, but in general accounts for between 5 to 20% of the total lytic activity. The reason for this

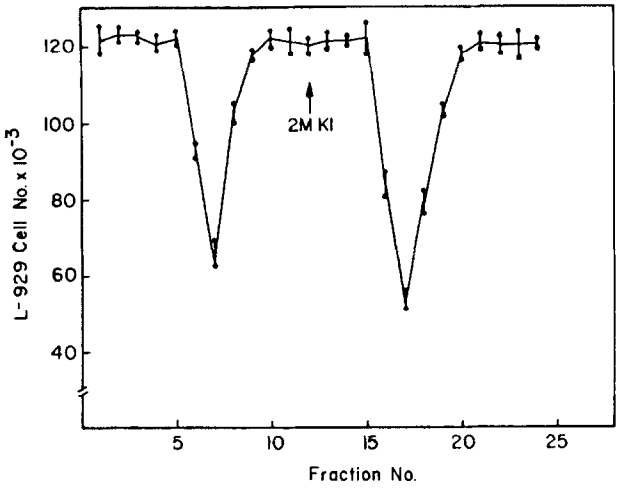

FIG. 6. Removal of complex LT activity on rabbit anti-human Fab'$_2$ affinity columns. A. Two milliliters of rechromatographed LT complex (the same sample as applied to Ultrogel AcA 22, shown in Fig. 4A) was applied to a 4 ml anti-human Fab'$_2$ affinity column equilibrated in PBS containing 2.0 mg IgG/ml beads, as described in *Methods*. The aliquot was allowed to incubate on the column for 30 min at 25°C, and was then eluted with four column volumes of PBS. Four milliliters of *2 M* KI was then eluted through the column, and the fractions containing KI were dialyzed against 1000 volumes of PBS at 4°C. All fractions were then tested for toxic activity by adding 100 μl to duplicate tubes containing 10^5 mitomycin-C treated L-929 cells for 24 hr at 37°C.

variation is not yet completely understood, but appears to be related to such parameters as the presence or absence of serum in culture, the type of stimulating agent employed (mitogen or antigen), and the type of lymphoid cell stimulated. Extensive studies have also verified that the appearance of complex is not due to a nonspecific aggregation phenomenon, for it was detected under many different kinds of culture conditions, and various attempts to form such a nonspecific aggregate out of α, β, and γ classes by concentrating them together in different conditions in the absence of complex failed to produce such a high molecular weight material. In fact, we find when fractions of α-LT are concentrated past a critical point, they form aggregates which are inactive. However, the smaller MW α-LT classes can associate with complex, when together in a crude supernatant, to form active complexes under conditions of low ionic strength. This latter observation suggests the smaller MW LT classes may associate with the larger MW complex class only in the presence of some form of condensing protein or "core molecule". This concept is also supported by the finding that smaller MW α-LT classes are released from the complex classes under conditions of high ionic strength.

Physical-chemical and immunologic studies of the human LT complex indicate the material is heterogeneous and is composed of several different molecular weight forms. This became evident when LT complex was subjected to velocity sedimentation on sucrose gradients or gel filtration on Ultrogel AcA 22. Cytotoxic activity eluted from these columns with a polydisperse profile, indicating that this material can exist in various forms having different molecular weights. LT complex activity was separated into four discrete molecular weight classes at 1×10^6 daltons (termed Cx1), 8×10^5 daltons (Cx2), 5×10^5 daltons (Cx3), and

1 to 2×10^5 daltons (Cx4). The relationship of these high MW classes one to another is not yet clear. However, it appears that these materials are related to the smaller α-LT forms, because at high ionic strength (0.5 *M* NaCl), components present in the complex class dissociate and elute as low MW α-LT classes. We have also observed on occasion, β and γ-LT components dissociating from the complex class under conditions of high ionic strength. These findings suggest that the complex class contains molecules that can interact together to form discrete units of different size. Moreover, these materials are apparently joined together by noncovalent bonds, because they are dissociated by relatively mild treatment(s).

Immunological studies also indicate that the complex class of human LT activity is composed of the smaller molecular weight LT forms, associated with some form of "core" molecule. This concept was supported by the observation that antisera directed against the various low MW classes (α and β) or subclasses (α_1 or α_2) of human LT molecules strongly inhibits the high molecular weight complex activities. How these smaller molecular weight LT forms assemble into forms of high molecular weight is not yet understood. However, we feel they must associate around a "core molecule", because: a) low MW classes will not convert to lytically active high MW forms when concentrated together under various conditions in the absence of complex; b) isolated low MW subclasses do not spontaneously form high MW classes; c) low MW classes will associate with complex under conditions of low ionic strength. We feel these "core molecules" may represent non-lytic components, which the smaller MW LT classes and subclasses could associate with. Indeed, it is conceivable that neutralization of complex activity by anti-class or subclass sera occurs by interaction of antibodies against these core molecules. This question cannot yet be resolved until further biochemical studies elucidate the nature of these core molecules. These complexes may also be heterogeneous with regard to the particular class(es) of small molecular weight LT components they contain. An observation to support this concept is that antisera directed against certain individual LT subclasses, i.e., α_1 and α_2, only neutralized a certain percentage of the total LT complex activity. These latter findings suggest the possibility that: (1) there may be forms of complex that are composed of different subunits; or (2) that each complex carries the same molecular composition, but that some subunits are internal and therefore not available to interact with anti-LT antibodies. Indeed, this latter situation has been observed in the formation of the large molecular weight terminal lytic unit of the complement system (10). The finding of discrete MW forms within the complex class indicates there may be dissociation of the largest subclass (Cx1) to the smaller subclasses by a regular breakdown through intermediate forms.

The present data reveal an important new finding, namely, that LT molecules, in the complex form, can interact with immunoglobulin-like material(s). We feel this is important, for it may provide the means whereby socalled "nonspecific cell-toxins" could interact with specific antigen binding receptor materials, such that their lytic activity could be specifically directed. This concept was suggested by the finding that antibody which would react with human Fab'_2 sites would neutralize a significant portion of complex class activity, while not affecting the smaller α, β, or γ classes of LT. Moreover, preliminary experiments suggest anti-Fab'_2 sera is most effective in neutralizing the Cx2 and Cx3 complex subclasses. These

findings strongly indicate that only certain forms of LT complex can associate with Ig-like molecules. However, the precise nature and specificities of the Ig molecules associated with LT complex are currently under study. A relationship between human LT molecules and immunoglobulin has been noted before. Amino, *et al.,* reported that a small percent of cytotoxic activity obtained from a continuous human lymphoid cell line could be inhibited with various anti-immunoglobulin serum (9). These findings could be explained by the capacity of a certain form of LT in a complex configuration to associate with immunoglobulin-like material. However, the possibility still cannot be ruled out that the LT molecules themselves may form some kind of antigen-specific binding site when they are together in the complex form. It is conceivable that this binding site may actually represent the core molecule around which other LT molecules may aggregate to form the complex class.

These studies suggest that antigen stimulated immune lymphoid cells could release LT molecules which are associated with specific antigen (Ig?) binding receptors. This indeed appears to be the case, since we find that a certain percentage of LT activity in supernatants from antigen stimulated immune human lymphocytes binds to affinity columns to which specific antigen is convalently attached (11). These materials can also complex with other molecules, for they can be expressed on the surface of lectin activated T cells (12) and will bind to L-cell surfaces *in vitro* (13, 14).

It is not yet clear which form of LT is the most biologically active one in causing cell lysis *in vitro*. It is clear that all forms can be detected on LT sensitive L-929 target cells *in vitro*. However, it is possible that L cells are uniquely sensitive to *all* the various MW forms of LT, whereas, other cells may only be sensitive to *certain* forms. Indeed, preliminary data suggests that when sephadex column fractions are tested on various cell types, the most effective form is the high MW LT complex class. However, it is entirely possible that LT in different MW forms would be more effective against one particular cell type than another. This heterogeneity of LT forms might provide a cell with a spectrum of lytic molecules which could destroy many different types of cells. It is difficult to conceptualize which form of LT is most effective in causing cell lysis without additional information, however, there are at least two possibilities, namely that LT molecules are synthesized as a high MW class, and by disassembly, result in formation of very active smaller MW classes, and the alternative, the smaller MW classes are assembled to form a highly active large MW lytic complex. The situation may be further complicated, since these reactions may occur both in the soluble phase, or on cell surfaces, and may involve different releasing lymphoid cell types.

There are certain analogies about the LT system which can be drawn from previously studied cell lytic systems. The first analogy can be drawn from the complement system, in which it is clear that the actual lytic unit is a complex of about 1×10^6 MW, formed by the coalescence of smaller subunits, which binds to the cell surface and induces lysis. These components are assembled in a cascade fashion from nonactive or weakly active subunits, requiring steric interaction of all the individual units to generate a highly active lytic complex (10, 15). Various bacterial toxins, particularly the α and β toxins of staphylococci, exist in various subunit forms that are able to interact with one another to form complexes, which can then interact with cell surfaces to cause lysis (16). In both these lytic systems,

there exists physical and immunological heterogeneity within the individual components. For example, physical heterogeneity has been demonstrated for the C'_2 component of human and guinea pig complement. These molecules appear to be immunologically identical, yet migrate on polyacrylamide gel as several distinct (isomeric) forms (17). A similar situation exists for the α and β components of staphylococci α toxins (16). While these two components are physically similar, they have distinct immunological reactivities. Moreover, they can assemble in various ratios to form polymeric complexes, some of which are lytically active and others which are not. It is possible that the LT system functions in a similar fashion, namely, that the smaller subunits may represent a less active form than the complex, which through the interaction of the component parts, perhaps in conjunction with an antigen binding receptor, are able to generate a highly lytically active unit, more active than the individual activities of the subunits themselves. This concept is also supported by the finding that the smaller MW forms, β- and γ-LT are highly unstable (18).

The role of lymphotoxins as effector molecules in lymphocyte mediated lysis of cells *in vitro* is at present not clear. However, that these molecules do play a role is becoming more convincing. Several recent reports indicate that antibodies specifically able to neuralize their activity *in vitro*, will block at least certain forms of these lytic reactions (19, 20). The present series of studies may indicate the difficulty which may be encountered in these types of investigations, which employ antibodies against various LT classes. First, it is clear that with the exception of a polyspecific antisera, it is possible to generate a sera which would not neutralize all forms of LT activity, and second, if a lytic complex is involved, it is possible that antibodies directed against individual components in the soluble phase may not be able to neutralize the activities of the materials once in a complex form, and finally, it is conceivable that antibodies, even against the subunits, would not be capable of neutralizing the complex once bound to a target cell surface. Indeed, this concept is supported by the finding that antibodies against the early components of complement are not effective in neutralizing the terminal lytic complex, once it is bound to the cell surface (21). It is clear, however, that in order to understand the role of these materials as lytic effectors in cell mediated immunity will require careful study, for the LT system is more complicated than previously suspected.

The present studies indicate that lymphotoxins comprise an interrelated system of cell-toxins. These molecules are able to interact with one another and other proteins to exist in various MW forms. Moreover, these various forms of LT molecules can exist in both the soluble phase and on lymphoid cell surfaces, possibly associated with antigen binding receptors. The present findings strongly negate the possibility that the activity of these materials is due to nonspecific factors, such as lysosomal enzymes. That this is a system of cell-toxins which has been conserved through evolutionary mechanisms is also supported by the finding that similar, although not as heterogeneous, classes of LT have been identified within various animal species (22).

Although complex, we feel soluble phase human LT molecules may represent the terminal phases of a complex sequence of events which ultimatly lead to cell lysis. Moreover, this system of molecules may be carefully regulated by the mechanism(s) involved in lymphocyte activation and/or delivery of the lethal event leading to cell lysis. Thus, these molecules could participate as cytotoxic

agents in both specific and nonspecific cell lytic reactions *in vitro*, depending upon the particular form in which they may exist, or how a particular lymphoid cell carries and delivers them during the lytic reaction.

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