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The Human LT System[1]

II. Immunological Relationships of LT Molecules Released by Mitogen Activated Human Lymphocytes *in Vitro*

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Materials with LT activity present in supernatants from PHA stimulated human lymphocytes *in vitro* are very heterogeneous and can be separated into multiple molecular weight classes, termed complex, α, β, and γ. Several of these classes can be further resolved into subclasses by other physical and chemical methods. The immunologic relationships of these materials one to another were examined employing various rabbit anti-humn LT sera which will neutralize LT activity on L-929 cells *in vitro*. These studies reveal: (a) LT activities are due to a distinct group of substances which are immunologically related one to another and can exist in several molecular weight forms; (b) a high MW class of molecules, termed complex, appears to contain all currently known LT classes and subclasses; (c) LT classes and subclasses both have common (public) and discrete (private) antigenic specificities; (d) human LT classes and subclasses do not appear to share Ag determinants with materials with LT activity released by lectin stimulated lymphoid cells from rabbit, rat, hamster, guinea pig, or mouse; and (e) human LT molecules are not immunologically related to cell toxins released by glass adherent human peripheral blood monocytes or PMN cells. These data indicate human LT molecules form a "discrete system" of lymphocyte derived cell toxins, which can associate together into various related but different MW forms in the supernatant.

INTRODUCTION

Activated lymphocytes can release materials, termed lymphotoxins (LT), which can cause growth inhibition or cell lysis *in vitro* (1). Physical-chemical studies reveal that materials with LT activity released by lectin activated human lymphocytes *in vitro* are heterogenous (2–5). While complex, these materials can be separated into four "classes" on the basis of their molecular weight, termed complex, α, β, and γ. These classes can be further resolved into multiple "subclasses" on the

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basis of their charge (5). However, the relationship of the classes and subclasses of human LT molecules one to another is still not clear. Antibodies are powerful tools when employed in conjunction with physical-chemical methods, to establish differences and relationships between macromolecules. Antibodies have been used to examine relationships between complex families of proteins, i.e., allotypes of immunoglobulin molecules (6), histocompatibility antigens (7), and C′ components (8). It is clear that generation of antibodies specific for human LT classes and subclasses would permit a means to begin to examine relationships of these materials one to another. Human LT molecules are immunogenic, for several investigators have generated antiserum in rabbits which will neutralize LT activity *in vitro* (9). However, to generate these antisera, these authors employed relatively large amounts of LT and immunized their animals over a long period. We have reported methods which only require small amounts of material for inducing antibodies in rabbits and goats, which will neutralize different classes and subclasses of human LT molecules *in vitro* (10). The present study employs these sera to examine the immunologic relationship between the various classes and subclasses of human LT molecules released *in vitro* by mitogen stimulated human lymphocytes. We find materials with LT activity in the supernatants to be composed of members which are both immunologically similar and those which are immunologically distinct from one another. A very interesting finding is that the high molecular weight LT class, termed complex, appears to contain all the subclasses of the human LT system.

MATERIALS AND METHODS

Target Cells and Culture Media

Stock cultures of mouse α-L-929 fibroblasts were used as target cells, as previously described (11). These cells were maintained in 32-oz prescription bottles in 95% air, 5% CO_2, and passed biweekly. Culture media consisted of RPMI-1640 (Grand Island Biological Co., Grand Island, N.Y.), supplemented with 3% heat-inactivated (56°C, 1 hr) fetal calf serum (RPMI-S) (Grand Island Biological Co., Grand Island, N.Y.), streptomycin (100 μg/ml) and penicillin (100 U/ml).

Lymphotoxin Assays and Antibody Neutralization Tests

The details of these methods have been reported previously (12). Briefly, target L cells, 10^5 L cells in 1.0 ml, were established as monolayers in screw-capped tubes in RPMI-S, containing 0.5 μg/ml mitomycin C (Sigma, St. Louis, Mo.). After a 24 hr incubation at 37°C, the cells were used as follows:

a. *Determination of the number of LT units in a given LT fraction or supernatant.* Serial dilutions of LT-containing or control supernatants or column fractions in RPMI-S were added to duplicate L cell cultures and allowed to incubate at 37°C. After 24 hr, the remaining viable adherent cell number was then enumerated on a Model F Coulter Counter. One unit of LT activity is defined as that amount of material which destroys 50% of the target L cells (50,000). The reciprocal of the highest dilution which destroys 50% of the target L cells denotes the number of LT units/ml in a given supernatant or fraction.

b. *Antiserum neutralization studies.* Each serum was first tested to determine its effectiveness to neutralize a known amount of LT activity on L cells *in vitro.* One milliliter of culture medium containing 100 to 300 units of added LT activity was incubated with various amounts of test or control antisera or IgG fractions for 1 hr at 37°C and subsequently placed on duplicate L cell tube cultures for an additional 25 hr at 37°C. The percent neutralization of LT activity was determined by the following formula:

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

From these dose curves we determined the optimal amount of antisera necessary to neutralize this amount of LT activity. This amount of serum was then used on subsequent tests to indicate the reactivity of that sera with other supernatants, classes or subclasses of LT.

Supernatant Production

A. *Human LT.* The details of these methods have been reported previously (13). Briefly, lymphocyte suspensions were obtained from tonsils and adenoids, and established in culture, at a density of 5×10^6 viable cells/ml in RPMI 1640, containing 20 μg/ml phytohemagglutinin-P (PHA, Difco, Detroit, Michigan) 20 μg/ml of a heat-stable boiled serum fraction obtained from newborn calf serum (BS) (13), and antibiotics. All supernatants from activated lymphocytes (SAL) were cleared of cells by either centrifugation at 300*g* for 10 min, or passage through one layer of glass fiber filter paper (Gelman, Ann Arbor, Mich.), in a Buchner Suction Funnel.

B. *LT activity from various animal species.* SAL obtained from various animal species was generated in the following manner. Details of this technique have been published elsewhere (14). Briefly, spleens from nonimmunized animals were aseptically removed, minced, and a single cell suspension prepared in RPMI-S. The cells were incubated in 32 oz glass prescription bottles at 37°C for 60 to 90 min after which the nonadherent cell population was removed and used as a source of lymphocytes. 25×10^6 of these cells, 95 to 98% viable, in 10 ml of RPMI-S were then placed in 36 mm Falcon plastic petri dishes. Each dish contained 5×10^5 mitomycin C treated L-929 monolayer cells, previously coated with PHA-P (100 μg/ml for 1 hr at 37°C). The lymphocytes and target L cells were allowed to incubate at 37°C for 8 to 10 hr, after which the cell-free supernatants were collected and tested for LT toxic activity. Neutralization of toxic activity in these supernatants by anti-LT was then tested.

C. *Toxic activity obtained from cultures of glass adherent human peripheral blood cells.* Freshly drawn heparinized peripheral blood was obtained from normal human donors and layered onto a 3% dextran PBS solution and allowed to sediment at 1*g* at 37°C for 1 to 2 hr. The leukocyte enriched layer was then removed and washed three times with PBS containing 3% FCS, and suspended in 20 ml RPMI 1640 containing 10% FCS at a density of 5×10^6 cells/ml. The cells were allowed to incubate at 37°C for 1 hr in 16-oz glass prescription bottles. Nonadherent cells were removed by gentle agitation and 20 ml of RPMI-S was added to the remaining adherent cells. After 36 hr at 37°C, supernatants from the glass adherent

cells were collected and cleared of remaining cells by centrifugation at 500*g* for 10 min. The supernatants were then stored at −20° until use in the neutralization studies.

Separation of LT Molecules for Immunization and Ab Neutralization Studies.

Human lymphocyte supernatants were first concentrated by a factor of 30 or 50 by passage through a BioRad Hollow Fiber Concentrator with a 30,000 MW exclusion size at 4°C (BioRad, Los Angeles, Calif.). The concentrate was aliquoted and frozen at −70°C, and/or subjected to a sequence of various physical-chemical separations employing a combination of molecular sieving, ion exchange, and gel electrophoresis, as previously described (5). Samples containing β or γ class LT activities were handled rapidly and tested as soon after collection or fractionation as was possible, because of their lability (15).

Immunization procedures and collection of antiserum

The details of these procedures are described elsewhere (10). All experiments employed outbred female New Zealand white rabbits (Valley Lab Supply Co., Mira Loma, Calif.), 2.5 to 3.0 Kg in weight. Samples for injection were emulsified in an equal volume of Freunds Complete Adjuvant (FCA). Acrylamide gel samples were first macerated by passage through a 3.0 ml syringe with a 20 gauge needle. Animals were immunized by three basic routes—subcutaneous (SC), intralymph node (IN), and intradermal (ID). The former were administered by the method of Vaitukaitis (VK-ID) *et al.* (16). Animals were bled by cardiac puncture 6 weeks after the initial immunization, and whole blood was allowed to clot at 37°C for 2 hr. The supernatant was carefully aspirated and cleared of RBC by centrifugation at 500*g* for 15 min. Cell-free serum was heated at 56°C for 45 min, filter-sterilized and frozen at −20°C in 7.0 ml aliquots. Preimmune serum was collected from each rabbit. Sera were tested for reactivity with the immunizing LT preparation, and if a positive response occurred in 6 to 8 weeks, these animals continued to receive immunizations. Only sera obtained from individual positive responder animals were pooled and employed in these experiments. Sera employed in these studies were obtained from animals immunized with one of the following preparations: (a) unfractioned serum-free, whole supernatants (anti-WS); (b) a single MW class of LT (anti-α, β, etc.) that had been purified by Ultrogel chromatography twice with an undetectable amount of contaminants from other classes: (c) a single highly refined LT subclass (anti α_1, α_2, etc.) that had been purified by Ultrogel chromatography twice, DEAE-cellulose chromatography once, and then subjected to PAGE. The animals were then immunized with materials obtained from PAGE gels.

Preparation of IgG fractions

A 40% ammonium sulfate precipitation (ppt) was performed on test or control serum at 4°C. The PPT was dissolved in PBS. The samples were then dialyzed against low salt DEAE buffer (0.05 *M* NaCl, 0.01 *M* Tris, pH 8.0) for 24 hr at 4°C. The dialyzed material was then ultracentrifuged at 10,000*g* for 30 min. The supernatants were then chromatographed over a DEAE column (2.4 × 20 cm) in low salt DEAE buffer. Each fraction was monitored for absorbence at 280 nm,

and the first peak of absorbency was denoted as IgG in all these studies. Double diffusion and immunoelectrophoresis of this fraction against goat anti-rabbit serum and goat anti-rabbit IgG revealed single bands of protein. Polyacrylamide gels of this fraction revealed a single broad band at the IgG region.

RESULTS

The Ability of Sera from Normal and Immunized Rabbits to Inhibit LT Induced Cytolysis of L-929 Cells In vitro

All sera were first tested to determine their effectiveness in neutralizing various standard human LT preparations *in vitro*. In these tests, the amount of LT was held constant at 100 to 300 units/ml and increasing amounts of serum were added to 2 to 3 ml samples. After 0 to 2 hr incubation at 37°C, 1.0 ml samples were tested in duplicate for LT activity, as described in *Methods*. An experiment which illustrates the data obtained by this technique is shown in Table 1. Antiserum from rabbits immunized with unfractioned whole supernatant (previously termed D series) and α class (termed B series) LT were tested in their ability to neutralize fresh whole supernatant LT activity or α class LT activity (10). All levels of test sera employed had inhibiting effects on *in vitro* LT-lytic activity. Maximum inhibition was attained with 50 to 150 μl of antiserum per ml of supernatant or α-LT. In contrast, preimmune or normal serum controls had little or no effect (0 to 10%). These experiments were repeated a total of three to five times with similar results. These sera were assigned a specific activity based on the number of LT units neutralized per microliter of serum. It was routinely observed that all sera employed in these studies possessed specific activities which ranged from 1 to 8. In these experiments, we thus employed antiserum of predetermined effectiveness against LT samples of activity which were preadjusted to 100 to 300 units/ml. Extensive physical-chemical and immunologic experiments were conducted with each antiserum or antiserum pool and have been reported elsewhere to verify that the LT neutralizing activity was due to interaction with immunoglobulin molecules and not by other mechanism(s) (9, 10, 17).

TABLE 1

The Capacity of Various Amounts of Anti-WS and Anti-α-LT Sera to Neutralize LT Activity Present in Whole Supernatants or Fractions of α-LT

Serum tested/ml of sample Anti-WS	LT preparation WS	LT preparation α-LT	Serum tested/ml of sample Anti-α-LT	LT preparation WS	LT preparation α-LT
50 μl	80[a]	70[a]	50 μl	30[a]	70[a]
100	100	95	100	40	95
200	100	100	200	60	100
300	100	100	300	65	100

[a] Increasing amounts of test and control sera were added to 2 ml samples of either whole supernatant (WS) or sephadex fractions containing alpha class LT activity. They were incubated 1 hr at 37°C and duplicate samples tested directly on L cells as described in *Methods*. The percent of neutralization was determined in comparison with the effects of similar amounts of normal or preimmune serum controls as described in *Methods*.

TABLE 2

The Capacity of Serum (Anti-WS) from Rabbits Immunized with Unfractionated Human Lymphocyte Supernatants to Neutralize Various Classes and Subclasses of Human LT Activity *in Vitro*[a]

Antiserum employed	LT preparation									
	Whole supernatant	Class				Subclass				
		Complex LT	α-LT	β-LT	γ-LT	α_1-LT	α_2-LT	α_{2a}-LT	α_{2b}-LT	α_3-LT
Anti-WS	+++[b]	+++	+++	+++	+++	+++	+++	+++	+++	+++

[a] Female New Zealand white rabbits were injected intradermal-Vaitukaitus (ID-VK) with whole supernatant (WS) prepared in serum free media from PHA stimulated human lymphocytes. These animals (designated D Series) were injected ID-VK once every 14 days for 8 weeks. Sera from each animal was then collected and tested for neutralization of LT activity in WS. Responder animals (designated D2 and D4) were subsequently injected subcutaneously with WS 20 days apart. Sera from individual rabbits obtained at the peak of their response, and all bleeds thereafter were pooled and employed for these experiments.

[b] Neutralization refers to inactivation of 200 to 300 units of LT activity by 100 μl of antisera calculated by the formula shown in *Methods*. − = 0 to 15% neutralization; + = 15 to 40% neutralization; ++ = 40 to 80% neutralization; +++ = 80 to 100% neutralization; N.T. = Not tested.

TABLE 3

Reactivity of Various Anti-LT Class Antisera with LT Activity in Whole Supernatant and Fractions Containing Various Classes or Subclasses of Human LT Activity *in vitro*

Antiserum employed	LT preparation									
	Whole supernatant	Class				Subclass				
	WS	Complex	α	β	γ	α_1	α_2	α_{2a}	α_{2b}	α_3
Anti-complex[a]	+[d]	++	+++	+	−	++	+	+	NT	−
Anti-α LT[b]	++	+++	+++	+	−	+++	++	+++	NT	++
Anti-β LT[c]										
F_1	NT	+++	++	++	+	++	++	NT	NT	++
F_2	NT	+++	−	++	−	−	−	NT	NT	-

[a] Rabbits were immunized by the VK-ID method with rechromatographed Ultrogel fractions containing complex LT activity in FCA. Sera were collected and tested as described in Table 1.

[b] Rabbits were immunized by VK-ID with rechromatographed Sephadex G-150 fractions containing α-LT. These rabbits (designated B series—5 rabbits) all responded to immunization. Sera collected from these five rabbits were then pooled together and employed in these experiments.

[c] Rabbits were immunized by VK-ID with rechromatographed Ultrogel AcA-44 fractions containing β class LT activity. The sera collected from these rabbits (designated F series—F_1 and F_2) was tested for reactivity with various classes of LT as described in *Methods*.

[d] Percentage neutralization calculated as in Table 2.

Capacity of Serum from Rabbits Immunized with Whole Supernatant (WS) from PHA Stimulated Human Lymphocytes to Neutralize WS and Fractionated Classes and Subclasses of Human LT Activity in Vitro

Antiserum employed in these studies was obtained from rabbits 2 and 4 of the D series which had been immunized with whole supernatant, as previously described (10). These sera were tested for their ability to neutralize various classes and subclasses of human LT activities. These experiments were conducted over a one year interval, and were repeated a total of five times with the fractions obtained from three to five different physical-chemical separations. The results of these studies are shown in Table 2. It is clear that these sera were effective in neutralizing from 80 to 100% of the lytic activity associated with whole supernatant and various LT classes and subclasses *in vitro*.

The Capacity of Serum from Rabbits, Immunized with Various Molecular Weight, Classes of Human LT, to Neutralize WS and Various LT Classes and Subclasses in Vitro.

Serum was obtained from animals immunized with the complex, α and β molecular weight human LT classes obtained by molecular filtration chromatography. Anti-complex sera employed are from two animals pooled separately. Both sets of sera gave similar patterns of reactivity. Anti-α serum employed is from a single serum pool of five responder animals which has been previously described (B pool). Anti-β serum employed is from two responder animals designated in the F series. The sera from each animal were separately pooled. These studies were repeated a total of three to seven times on materials resulting from numerous supernatant lots and separations. These collected results are shown in Table 3. Anti-complex sera was strongly reactive with complex, α class, and α_1 subclass, and weakly reactive with WS, β class, and α_2 and α_{2a} subclasses. It was nonreactive with γ class and α_3. Anti-α serum was strongly reactive with WS, complex, and α class and all α subclasses. It was weakly reactive with β class and nonreactive with

TABLE 4

Reactivity of Anti-Subclass Antisera with LT Activity in Whole Supernatants, and Fractions Containing Various Classes or Subclasses of Human LT *in Vitro*[a]

Antiserum employed	LT preparation											
	Whole supernatant	Class				Subclass						
		Complex LT	α-LT	β-LT	γ-LT	α_1-LT	α_2-LT	α_{2a}-LT	α_{2b}-LT	α_3-LT	β_1-LT	β_2-LT
Anti-α_1-LT	+[b]	++	+	−	−	++	−	−	NT	−	NT	NT
Anti α_2-LT	+++	++	++	+	++	+++	+++	+++	NT	++	NT	NT
Anti-β_2 (F_1)[c]	NT	+++	++	++	+	++	++	NT	NT	++	−	++
Anti-β_1 (F_2)	NT	+++	−	++	−	−	−	NT	NT	−	++	−

[a] Subclasses of human α-LT obtained after ion exchange chromatography and PAGE were injected by the VK-ID technique into each animal, as described in Table 2. One rabbit received preparations containing α_1-LT, and one rabbit received preparations containing α_2-LT. Both rabbits responded by 8 weeks. The antisera employed for these studies were collected after 8 weeks.

[b] As in Table 1.

[c] Sera from rabbits F_1 and F_2 (anti-β-LT class) were obtained as described in Table 3.

TABLE 5

Reactivity of Anti-Whole Supernatant (Anti-WS) with LT Activity in Supernatants Obtained from PHA Stimulated Lymphoid Cells from Various Animal Species *in Vitro*[a]

Species studied	Units/ml + NRS	Units/ml + Anti-WS	% Neutralization
Human	250 ± 22	<2	>99%
Mouse (C57B1/6)	325 ± 16	310 ± 23	N.S.[b]
Rat	16 ± 1	18 ± 1	N.S.
Hamster	28 ± 3	35 ± 4	N.S.
Guinea Pig	96 ± 7	91 ± 3	N.S.
Rabbit	270 ± 14	256 ± 10	N.S.

[a] LT activity from various animal species (except human) was obtained from non-glass adherent splenic lymphocytes by incubation in petri dishes containing PHA coated L-929 cells in RPMI, 5% FCS, for 8 to 10 hr at 37°C, as described in Methods. Cell-free supernatants were collected and tested for toxic activity by determining the number of LT units/ml, as described in *Methods*. Results are expressed as the units LT/ml in the presence of 100 μl NRS or 100 μl rabbit anti-WS antisera. Percent neutralization was calculated as described in *Methods*.

[b] N.S. = Not significant.

γ class LT activity. Anti-β class serum from animals F_1 and F_2, gave distinct neutralization profiles. Serum from the former reacted strongly with all LT classes and subclasses, except γ class. Whereas, serum from F_2 reacted strongly with all but α-LT class and subclasses. These data indicate there are immunologic relationships and differences between the various classes and subclasses of LT molecules.

The Capacity of Serum from Rabbits Immunized with Different Human LT Subclasses to Neutralize Separated Classes and Subclasses of LT Activity in Vitro

Serum was obtained from animals immunized with highly refined preparations of α_1 and α_2 human LT subclasses. These LT preparations were obtained as described in *Methods*. Antiserum reactive with the different β-LT subclasses was obtained from rabbits which had actually been immunized with β class fractions. However, upon testing, they were found to be reactive with the different β subclasses, β_1 and β_2. These experiments, shown in Table 4, were repeated a total of three to four times and involved testing samples from several different separations on various supernatant pools. Anti-α_1 subclass serum reacts with complex and α class, but does not react with β or γ class. Anti-α_1 also neutralizes α_1 subclass but not α_2 or α_3. Anti-α_2 serum reacted strongly with all classes and subclasses except β class. Antisera from a rabbit (F1) immunized with β class LT was strongly reactive with complex, α class, α subclasses, and β_2 subclass. However, it was moderately reactive with γ class and nonreactive to β_1 subclass. Antisera from a second rabbit (F2) immunized with β class LT was strongly reactive to β_1 subclass and complex, but nonreactive with γ, α, and α subclasses, and nonreactive with β_2 subclass. This is clearly a complex pattern of immunologic relationships.

The Capacity of Polyspecific Anti-Human LT Serum (Anti-WS) to Neutralize LT Activity Released by Lectin Stimulated Splenic Lymphoid Cells Obtained from Various Animal Species in Vitro

Media were collected from PHA activated cultures of spleen cells obtained from various animal species as described in *Methods*. Test and control media were as-

sessed for the presence of LT activity. In a number of experiments, the spleen cell suspensions were exposed to glass surfaces to remove adherent cells. Test and control media were diluted serially and to each duplicate set of tubes was added normal rabbit serum or polyspecific anti-whole supernatant serum from rabbits D_2 or D_4. Percent neutralization was calculated as described in *Methods*. The results of two separate sets of experiments are shown in Table 5. It is clear this antiserum had no effect on the LT activity released by lectin stimulated cells from these various animal species.

The Effect of Various Anti-Human LT Sera on the Lysis of L-292 Cells in Vitro Induced by Supernatants from Glass Adherent Human Peripheral Blood Leukocytes

Culture supernatants were collected from glass adherent cell populations obtained from normal peripheral blood leukocytes as described in *Methods*. These supernatants were tested for toxic activity in the presence of normal rabbit serum and various anti-LT serum. Previous tests indicated these supernatants possessed levels of toxic activity in the range of 20 to 35 units/ml as measured in the standard assay. These experiments were repeated twice with duplicate tubes for each serum. The results are shown in Table 6. It is clear that only anti-WS was reactive with these lytic activities.

DISCUSSION

There were several types of anti-human LT sera employed in the present studies. The first type, rabbit antisera obtained from animals immunized with unfractionated cell-free lymphocyte supernatants, termed anti-whole supernatant (anti-WS). The second type, termed anti-class sera, is obtained from animals that have been immunized with rechromatographed ultrogel fractions containing all members of a single MW LT class, i.e., complex, α and β. Finally, the last type, more specific sera, obtained from animals which were immunized with carefully fractionated preparations containing LT subclasses. It should be emphasized that none of the LT preparations employed in these studies has been tested for its degree of chemical purity. However, the characteristics of each preparation with regard to its containing various

TABLE 6

Capacity of Various Anti-Human LT Sera to Neutralize Toxic Supernatants Obtained from Glass Adherent Human Peripheral Leukocytes *in Vitro*[a]

Anti-whole supernatant	Antiserum employed			
	Anti-class		Anti-subclass	
	Anti-complex	Anti-α	Anti-α_1-LT	Anti-α_2-LT
++[b]	−	−	−	−

[a] Buffy coat leukocytes were collected from human peripheral blood and glass absorbed at 37°C for 1 hr. Nonadherent cells were removed by gentle agitation, fresh media was added to the culture of adherent cells, and the cultures were allowed to incubate at 37°C for 36 hr. Supernatants were collected and cleared of cells by centrifugation and stored at −20°C until used in these studies.

[b] As in Table 2.

LT classes or subclasses has been carefully defined (5). These preparations thus could be referred to as functionally purified with regard to the various LT molecules. Thus, antisera obtained from animals immunized with these preparations can be employed to distinguish immunologic relationships and differences between classes and subclasses of human LT.

Specific experiments verified that the *in vitro* neutralizing ability of these sera, as indicated on L-929 target cells, was due to immunoglobulin molecules and not to other nonspecific effects, such as enzymatic degradation, etc. (9, 10, 17). Two types of *in vitro* LT neutralization tests were employed in these studies. The first, in which LT was held constant and increasing amounts of serum were added. This method provided a means to measure the effectiveness of a certain volume of sera to neutralize a certain number of units of LT activity. The second assay was used for screening and, once known how effective a sera was in its ability to neutralize LT activity, a set volume was tested against various LT fractions. This latter method was used to compare the ability of a single sera to neutralize other classes and subclasses of human LT molecules with known amounts of lytic activity. The exact mechanism of antiserum neutralization is not known. However, there are two possibilities: (1) that these antisera aggregate the soluble phase LT molecules together, thus reducing the total number of cytotoxic units available to interact with a susceptible cell, or (2) they physically block or interact directly with the cytotoxic site itself and prevent cell lysis by direct steric hindrance. It is also not yet clear about the chemical nature of the antigenic determinant sites on the LT molecule(s) recognized by these various antisera. These sites are assumed to be protein, but we cannot exclude the possibility that these molecules possess small amounts of lipid or polysaccharide, which could be recognized as antigen determinant sites. Preliminary studies, however, favor the former possibility, since treatment of these molecules with neuraminidase does not affect their antigenicity (unpublished results).

The different classes and subclasses of human LT molecules are composed of members and submembers which are immunologically distinct. The differences between these substances first became obvious when we tested the capacity of anti-class or subclass sera to neutralize whole, unfractionated supernatants and found they could only neutralize portions of the total lytic activity. Indeed, the only antisera in the present studies that could neutralize all lytic activity in an unfractionated supernatant was anti-WS sera. The nature of these immunologic differences becomes more evident when we examine the capacity of anti-class and subclass sera to neutralize various classes and subclasses of LT activity. It has been previously reported that anti-α class human LT sera will not crossreact with β class LT activity (2), suggesting there are unique α and β class determinants. We also found this in several of our animals immunized with anti-α class sera (15), however, this was later found to be an exception rather than a general finding. Most anti-α class sera reacted at least weakly with β class LT molecules. The reason for this crossreaction will be discussed in a following paragraph. We also found that anti-α_1 LT subclass sera will not recognize any β LT subclasses. In addition, anti-β_1 LT will not recognize any α subclasses. These data suggest it is possible to obtain an anti-α subclass serum directed at α_1 subclass which would not recognize any β class activity. It was also clear that anti-α_1 LT sera did not recognize either α_2 or α_3 subclass. In addi-

tion, we found that antisera which recognized β_1 subclass did not apparently cross-react with β_2 subclass, and vice versa. Thus, human LT molecules can exist in a number of forms which are immunologically distinct from each other.

The present data also indicate that while materials with human LT activity are immunologically heterogeneous, there are varying degrees of immunologic similarity between the various LT forms. This first became apparent when we found those antisera which were reactive with members of the α and β classes and subclasses of human LT molecules were very effective in neutralizing the large molecular weight class, termed complex. In a similar fashion, anti-complex sera was reactive with members of the smaller molecular weight α and β LT classes, but did not react with γ classes. Other relationships became apparent when we examined the reactive patterns of the anti-subclass sera with separated LT subclasses. For example, anti-β_2 (stable) sera reacted strongly with all the other LT classes and all subclasses except β_1 (unstable). Whereas, anti-β_1 sera only reacted strongly with complex and β_1 LT subclass. Anti-α_2 serum reacted strongly with all the other LT classes and at an intermediated level, with β_2. These data clearly indicate that different human LT classes and subclasses share certain common antigenic determinant sites.

The above findings support the concept that, while heterogeneous, materials with LT activity released by lectin activated human lymphocytes *in vitro* form an interrelated system of cell toxins. A complete understanding of the individual members and interrelationships of the components of this system will require additional careful study at the molecular level. However, interpretation of the present data allow, with clear limitations, formulation of the following hypothesis. LT molecules form a system of cell toxins composed of a number of individual members which may associate with one another to form complexes of various MW. This concept is more strongly supported by the identification of all the small MW LT classes in the large MW complex class. How the various LT forms are generated and related one to another is not yet clear, but it appears they may result from the association or disassociation of different subunits. There may be certain subunit(s) which are common to a number of different LT forms, and others which are unique to single forms. This is supported by the finding that antigenic determinants expressed on β_2 subclass molecules appear to be expressed on all the other LT classes, including γ LT activity. In contrast, determinants expressed on β_1 subclass only appear in the high MW complex class. Clearly, β_1 and β_2 subclasses are physically (i.e., charge and stability) and immunologically unrelated but can associate together, and both appear in the complex class. Very little is known about the physical-chemical properties of the γ LT class, because of its instability, however, these studies show it is clearly immunologically related to and perhaps derived from the stable α and β_2 subclass. It is also possible that these molecules may be enzymatically derived from the more stable large MW LT forms, for the β_2 subclass has been shown to be inactivated by a lymphocyte-serum-dependent mechanism(s) (18). Thus, the smaller MW forms may represent materials on an inactivation pathway. The molecular basis of and the reason for the degree of physical heterogeneity among the human α LT class molecules is not clear. There are a number of physically resolvable α-LT subclasses with both similar and distinct antigenic determinants. Whether this represents micro-heterogeneity of closely re-

lated isomeric" forms of the same gene product, which was recently reported for the C'_2 component of the human complement system (19), will require more study. While each α subclass expresses common antigenic determinant sites associated with the β_2 subclass, α_1 also expresses unique determinants from those of α_2 and α_3. Physical-chemical and immunologic data suggest the subclasses α_1, α_2, and α_3 may represent related (all contain β_2 determinants) but heterogeneous (anti-α_1 sera does not react with α_2) molecules, whereas all the α_2 subclasses, i.e., 2a, b, c, d, etc., may represent closely related isomeric gene products.

Anti-LT sera will be powerful reagents in determining if these molecules play any roles as lytic effectors in various forms of cell mediated lytic (CML) reactions, both *in vitro* and *in vivo*. Several reports already indicate they are important effectors in several types of lymphocyte induced cell and tissue destructive reactions and may not be involved in other types (20, 21). They have also been identified *in vivo* (22). However, it must be emphasized that the present results, if they can be generalized to other animal species, indicate that unless the exact reactivity of an anti-LT serum is known, a negative result may not truly eliminate the role of these material(s) in CML reactions.

It appears that while heterogeneous, similar forms of human LT α class molecules are released by human lymphoid cells activated by different methods. It has been reported that rabbit anti-α class LT sera would neutralize LT activity from human lymphoid cells stimulated by co-culture with lectins, allogeneic lymphocytes (MLC) and cells from tuberculin positive donors cultured with PPD (9). In addition we found anti-α class or anti-β_2 subclass sera will totally neutralize LT activity detectable in supernatants from several continuous lymphoid cell lines (23). These cells did not release any LT molecules in the β or γ-LT MW form. However, they were broadly reactive anti-class sera and more careful examination of these activities with anti-subclass sera may reveal differences.

The LT system in humans appears to be a family of cell toxins which may be unique to lymphoid cells. In addition, while heterogeneous, LT molecules from other animal species do not appear to crossreact with antisera reactive with human LT molecules *in vitro*. Anti-human LT sera was not reactive with LT activities released by lectin activated lymphoid cells from rabbit, rat, guinea pig, or mouse. Our data extend similar observations reported by Walker and Lucas (9). The latter data collectively support the concept that these antisera are reactive with unique antigenic determinants associated with LT proteins, rather than common saccharide moieties. In addition, certain anti-human LT class and subclass sera did not react with cytotoxin(s) spontaneously released by glass adherent (GA) human peripheral blood cells. However, while we did not test anti-β class sera against these GA toxins, Walker *et al.*, found some crossreactivity with their anti-β sera. These findings are important and serve to distinguish lymphotoxins from previously reported toxic material(s) released from GA monocytes and polymorphonuclear cells (24). However, the polyspecific sera from animals immunized with unfractionated supernatant did partially react with toxin(s) released by GA cells. These results suggest the presence of some of the materials in unfractionated supernatants, but that they were removed upon fractionation. The present data support the concept that human LT molecules represent a discrete and "unique" system of lymphocyte derived cell toxins, which may associate with one another into various MW forms.

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