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THE LT SYSTEM IN EXPERIMENTAL ANIMALS

II. Physical and Immunologic Characteristics of Molecules with LT Activity Rapidly Released by Murine Lymphoid Cells Activated on Lectin-Coated Allogeneic Monolayers *in Vitro*[1]

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The present studies investigate the physicochemical and immunologic properties of cell-lytic molecules released *in vitro* by nonadherent C57/BL/6 splenocytes or nylon wool-enriched T cell populations activated on monolayers of PHA coated L-929 cells. The findings reveal cell-lytic molecules released by these lymphoid cells are physically heterogeneous. These molecules can be separated by gel filtration into similar m.w. classes previously observed for human LT molecules. Three major classes, termed complex (Cx) (>200,000 d), α heavy (α_H) (110 to 140,000 d)α light (α_L) (60 to 90,000 d), and two minor classes, β, at 40 to 50,000 d and γ at 10 to 20,000 d were observed. Chromatography of supernatants in high ionic strength buffers dissociates Cx and α_H to the smaller m.w. α_L form. This evidence suggests that Cx and α_H MW classes are physically related to the smaller m.w. α_L class. Fractionation of the α_H m.w. LT class by DEAE or PAGE resolved these molecules into additional distinct subclasses. Antisera were made against fresh serum-free whole supernatants (anti-WS) or rechromatographed Ultrogel fractions containing α_H molecules (anti-α_H). Anti-α_H and anti-WS react with *all* m.w. classes of murine LT molecules, indicating these various forms are immunologically related. These antisera do not react with LT molecules obtained from several other animal species or with "nonspecific" intracellular toxins, e.g., lysozomal enzymes, present in normal PMN or phagocytic cells.

These data indicate that materials with cell-lytic activity present in these culture supernatants are LT molecules, because: a) certain m.w. forms observed are similar to those reported previously, and b) these various m.w. forms are all physically and immunologically interrelated. These studies also indicate that murine LT molecules like human LT molecules are heterogeneous, but appear to comprise a system of subunits, in which the large m.w. form may dissociate into the smaller m.w. forms.

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Several studies have demonstrated that molecules possessing lymphotoxin (LT)[2] cell lytic activity released *in vitro* by activated lymphoid cells are physically heterogeneous (1–4). Human LT molecules have, to date, been the most widely studied, because they are present in a given lymphocyte supernatant in much higher activity, and culture techniques have been devised to help stabilize their lytic potential (4). Extensive physical studies on human LT molecules present in 3- to 5-day lectin-stimulated supernatants have demonstrated these materials can be resolved by gel filtration techniques into at least four major m.w. classes, termed Cx (>200,000 daltons, d), α (70 to 150,000 d), β (30 to 50,000 d) and γ (10 to 20,000 d), and several of these classes further resolved into multiple "subclasses" on the basis of overall molecular charge (1, 2, 5). Immunologic studies employing different types of anti-human LT antisera have indicated that many of the various m.w. LT forms *are* immunologically related (6) and that *all* m.w. forms appear to be present in the large m.w. complex class (6, 7). These observations have led to the hypothesis that the smaller m.w. human LT molecules (α, β, γ) comprise a "system" of subunits that can interact together to form high m.w. complexes (7). In addition, certain of the complex LT forms can associate with Ig or Ig-like receptor molecules that possess specific antigen-binding capacity (7–9). Recent studies indicate that the Cx m.w. LT forms possess dramatically increased *in vitro* cell lytic capacities when compared to the smaller m.w. (α, β, γ) LT forms (10).

Cell lytic (LT) activity released *in vitro* by activated murine lymphoid cells also appears to be due to materials that are physically heterogeneous. Although not as extensively studied as human LT molecules, murine LT molecules were reported in one study to have m.w. between 80 and 150,000 d (11), and in another 45,000 d (12). However, in the latter study, minor peaks of activity were also observed at >200,000 d and 150,000 d. The first manuscript of this series reported a new method for inducing rapid release of high levels of cell lytic (LT-like) activity detectable on L-929 cells from lymphoid cells obtained from mice and other animal species *in vitro* (13). Lytic activity in these supernatants was resolved into at least three major and two minor m.w. classes by gel filtration chromatography. Furthermore, resolution of the α_H LT class on DEAE and PAGE revealed multiple charge subclasses of these molecules. Additional studies indicate murine LT molecules are similar to

[2] Abbreviations used in this paper: LT, lymphotoxin; PHA/L-929, phytohemagglutinin-P coated L-929 fibroblasts; LT Cx, high m.w. (200,000) lymphotoxin complex; Anti-WS, rabbit anti-mouse whole supernatant; Anti-α_H, rabbit anti-mouse α_H LT (110-140,000 daltons) d, daltons; PAGE, polyacrylamide gel electrophoresis; s.c., subcutaneous; NRS, normal rabbit serum.

human LT molecules, and although physically heterogeneous, represent a system of related subunits, which can interact together to form high m.w. complexes.

MATERIALS AND METHODS

1. Target cells and culture media. Target cells and culture media employed were identical to the first manuscript of this series (13).

2. Production of LT-containing culture supernatants. The details of these procedures were reported in the first manuscript of this series (13).

3. Separation of C57BL/6 splenic T cells. Enriched T lymphocyte populations were obtained from nylon wool columns, as described by Julius (14). The details of the procedures employed, and the purity of these cells were described in the first manuscript of this series (13).

4. Lymphotoxin assay. These techniques are described in detail in the first manuscript of this series and elsewhere (13, 15).

5. Physicochemical separation of LT. The methods employed for fractionation of LT activity in supernatants from activated murine lymphocytes were identical to those employed for separating human LT molecules, all of which have been previously published (2). When used for physicochemical studies, fresh supernatants obtained from activated murine lymphoid cells were pooled into 100 to 500 ml lots, concentrated 25 to 50 × by ultrafiltration employing Amicon concentrators with PM10 membranes and immediately fractionated. All procedures were performed at 4°C.

A. Molecular sieving. Degassed Ultrogel AcA 44 (LKB, Uppsala, Sweden) was poured to a bed height of 95 to 100 cm in 2.5 x 120 cm siliclad-coated columns, equilibrated in low salt buffer, 10 mM potassium phosphate, pH 7.2, various concentrations of NaCl and 10^{-4} M EDTA. The columns were calibrated numerous times over the period of these studies with blue dextran (BDx—2 × 10^6 d), human IgG (IgG—150,000 d), hemoglobin (Hb—64,000 d), α-chymotrypsin (α-CT—23,000 d) and phenol red (PR—364 d), applied in a total volume of 2.0 ml. Six-milliliter fractions were collected at a flow rate of 24 ml/hr, and monitored for their absorbance at 280 nm on a Gilson Model 2000 Spectrophotometer (Middleton, Wis.). Two-milliliter samples of concentrated lymphocyte supernatants containing from 1000 to 5000 units of LT activity were chromatographed over these columns, and 0.05 to 0.2-ml aliquots of the eluted fractions were assayed for LT toxic activity. Rechromatography of the various LT classes was performed in a fashion identical to the original separation procedures.

B. DEAE-cellulose chromatography. DEAE (DEAE 11, Whatman, Clifton, N. J.) was washed, degassed, and poured to a bed height of 7 cm in a 10-ml plastic syringe and equilibrated in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA (DEAE buffer). Rechromatographed LT fractions from several molecular sieving columns were pooled, tested for LT activity, dialyzed extensively against 10 mM Tris, pH 8.0, 0.1 mM EDTA by pressure ultrafiltration on Amicon PM-10 membranes, finally concentrated to 2.0 ml, and applied to the column. Protein was eluted with 30 ml of 10 mM Tris, 0.1 M EDTA, followed by a linear 100-ml gradient from 0 to 0.3 M NaCl, DEAE buffer, followed by 30 ml of 1.0 M NaCl in the same buffer. Two-milliliter fractions were collected at a 20 ml/hr flow rate, tested for conductivity, and 0.05 to 0.10 ml was tested for LT activity.

C. Polyacrylamide disc gel electrophoresis (PAGE). PAGE was performed by the method of Davis (16). A 50 to 150-μl sample (containing from 5 to 50 units LT activity) in 20% sucrose was applied to a 0.5 × 8.0-cm gel column consisting of a 1 cm 3% acrylamide stacking gel and a 7 cm 7% acrylamide separating gel in 50 mM Tris-glycine, pH 9.6. The sample was subjected to electrophoresis at 4 mA/gel at 4°C. The gels were then cut into 2-mm slices, and each slice was incubated in 0.3 ml RPMI-S for 24 hr at 4°C. A 100-μl sample was then added to duplicate 1 ml L cell cultures and tested for LT activity. Rf values were calculated with reference to the migration of a bromphenol blue (BPB) marker.

6. Production and use of antisera. The methods employed for generation of immunogens, immunization procedures, and testing and handling of antisera used in these studies were identical to those reported for generation of anti-human LT sera (6, 17).

A. Anti-whole supernatant. Two rabbits were initially immunized intradermally by the method of Vaitukaitus *et al.* (18) with concentrated (20 ×) fresh, whole culture supernatants prepared in serum-free media and suspended in complete Freund's adjuvant (CFA). These supernatants contained from 1000 to 5000 units LT activity/ml, and 5 ml were injected. Subsequent to this injection, every 2 weeks up to 3 months, these animals received another 2 ml injection subcutaneously (s.c.) of concentrated fresh whole supernatant in serum-free media, and after 12 weeks, the animals were bled by cardiac puncture, the serum was collected and tested for LT-neutralizing activity. Sera used in these studies are from the 8th to 14th month post-immunization.

B. Anti-α heavy sera. Two rabbits were initially immunized as above with rechromatographed Ultrogel fractions of serum-free supernatants containing all subclasses of the 110 to 140,000 d α_H class of LT molecules, but free of other m.w. LT classes, suspended in CFA. The animals were then boosted bimonthly, and after 6 months they were bled and the serum was tested for LT-neutralizing activity. All sera, including preimmune or normal rabbit sera (NRS), were heat inactivated (56°C, 60 min) and clarified by centrifugation at 20,000 rpm for 30 min before use. Neutralization of LT activity was determined by incubating various amounts of test or control sera with 100 μl of LT-containing supernatants or Ultrogel fractions for 1 hr at 4°C, and testing the remaining LT activity on L-929 cells. Percent neutralization of LT activity was calculated by the following formula:

$$\% \text{ Neutralization} = \frac{\text{Cell No. + Ab} - \text{cell No. + NRS}}{\text{Cell No. + control media} - \text{cell No. + NRS}} \times 100$$

These various antisera were not toxic to L-929 cells by themselves when used at the levels employed in these experiments.

7. Preparation of IgG fractions and absorption of antisera. IgG fractions of rabbit anti-WS, anti-α_H, or NRS were obtained after DEAE chromatography of 40% $(NH_4)_2SO_4$ fraction of whole serum (19). When adsorbed on L-929 cells, or nonglass-adherent splenic lymphocytes, 1 ml of serum was incubated with various numbers of these cells for 1 to 4 hr at 4°C, after which the cells were removed by centrifugation, and the sera were clarified by ultracentrifugation at 20,000 × G for 30 min.

8. Preparation and use of immunoabsorbent affinity beads. Normal C57BL/6 mouse serum or newborn calf serum was covalently linked to cyanogen bromide-activated Sepharose 4B, according to the methods described by March *et al.* (20). Two milligrams of serum protein were bound per milliliter of beads in each case. One milliliter of antisera or NRS was allowed to incubate with 1 ml of packed beads in PBS at 4°C for 2 to 4 hr. The beads were then removed by centrifugation, and the sera

were collected and tested for LT-neutralizing activity as described above.

9. Homogenates of unseparated spleen cells from C57BL/6 mice. A single cell suspension containing 2×10^9 spleen cells in 2 ml RPMI 1640 was sonicated for 1 min with a Bransonic 32 Sonicator. Microscopic observations revealed 100% cell lysis had occurred. Large particulate matter was removed by ultracentrifugation at 20,000 × G for 30 min, and 200 μl of the resulting supernatant were tested for toxic activity on L-929 cells. "Toxic activity" in these preparations was low but generally contained between 1 to 3 units/ml 10^9 splenic leukocytes when tested on L-929 cells *in vitro*.

RESULTS

1. Stability of LT activity released in vitro by C57BL/6 spleen cells or purified T cells activated on lectin-coated allogeneic monolayers. To test the stability of these materials, supernatants were collected after 8 hr of lymphocyte-PHA/L cell interaction, and stored at various temperatures. Figure 1 shows that toxic activity present in supernatants from activated C57BL/6 NGA lymphocytes (Fig. 1*A*) or enriched T cells (Fig. 1*B*) rapidly decays when stored at 37 or 4°C. This activity can, however, be partially stabilized by storage at −70°C, but only for approximately 2 weeks. Although the majority of activity decays in 12 to 36 hr, 10 to 20% of the activity appears to be more stable at 4°C for 2 to 3 weeks. Additional experiments also revealed that the presence of serum in these cultures does not appear to alter the stability of these molecules. These

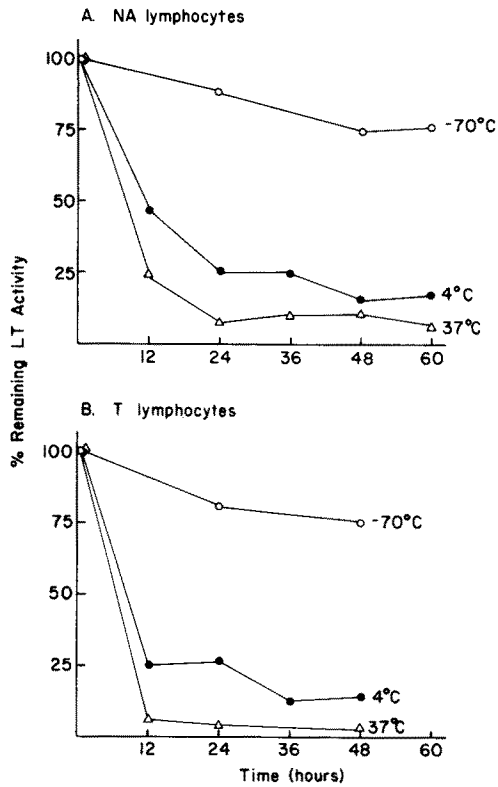

Figure 1. Stability of lytic activity present in supernatants from activated C57BL/6 nonadherent lymphocytes or purified T cells after storage at various temperatures. *A*, nonglass adherent C57BL/6 splenic lymphocytes were activated on monolayers of PHA-P-coated L-929 cells for 9 hr at 37°C, as described in *Materials and Methods*. Supernatants were collected and tested for lytic activity on L-929 cells either immediately or after storage at various temperatures for various periods of time. Initial lytic activity was 128 units/ml. *B*, same as *A*, except nylon wool-purified T lymphocytes were employed as the responding cells. Initial lytic activity was 86 units/ml.

experiments were performed three to four times with supernatants collected from C57BL/6, DBA/2, or C3H/DiSn lymphoid cells with identical results.

2. Resolution of various m.w. classes of LT molecules by gel filtration chromatography on Ultrogel AcA 44. Because of the instability of LT activity in these cultures, all fractionation studies were performed quickly and without intermittent storage of fractions. Supernatants were collected from cultures produced in serum-free media, immediately concentrated 20 to 50 × by ultrafiltration by using Amicon PM10 membranes, and subjected to gel filtration chromatography by employing Ultrogel AcA 44 in 0.01 M phosphate buffer with various concentrations of NaCl. Fractions were collected and tested for toxic activity on L-929 cells as described in *Methods*. Shown in Figure 2*A* is a representative elution profile of the toxic activity present in a single supernatant. However, similar profiles were observed in column separations performed on 40 to 50 different supernatant preparations. Clearly, LT activity present in supernatants from activated B6 NGA spleen cells is heterogeneous, but is resolvable into several distinct m.w. components. For the purpose of discussion, we have adopted the terminology employed for LT molecules isolated from human lymphoid cells (1). Individual peaks eluting off gel filtration columns define individual LT *classes*. Materials with LT activity eluting in the void volume (>200,000 d) are termed LT complex *class* (LT Cx), materials eluting in the 70 to 150,000 d range are termed *α class*, materials eluting in the 35 to 50,000 d range are termed *β class*, and materials eluting in the 10 to 20,000 d range are termed *γ class*. Thus, murine LT molecules present in these culture supernatants appear to be resolvable into a complex (>200,000 d), *two* α forms, and in several profiles, β and γ m.w. components were observed. These two α LT forms have been termed α heavy (α_H) (110 to 140,000 d) and α light (α_L) (60 to 90,000 d). Although there is variation in the amount of activity associated with a given m.w. LT class, α_H and α_L m.w. forms were the most consistently observed. The percentage of LT activity generally found to be associated with an individual LT class in different supernatant preparations varied as follows: LT Cx (0 to 20%), α_H (50 to 80%), α_L (5 to 30%), β or γ (0 to 10%). Furthermore, as is indicated in Figure 2A-C, it is clear that the ionic strength of the separating buffers can have dramatic effects on the apparent percentage of each class observed. Figure 2A-C shows a representative experiment in which a single supernatant was chromatographed over three separate but identical Ultrogel columns equilibrated in low salt, 0.15 M NaCl, or 0.5 M NaCl. We found that in low salt buffers, the predominant m.w. LT classes detected are in the Cx and α_H m.w. forms (Fig. 2*A*). As the ionic strength increases to 0.15 M NaCl (Fig. 2*B*) or 0.5 M NaCl (Fig. 2*C*), the predominant m.w. forms shift to the α_L m.w. component, with a concurrent reduction of Cx and α_H m.w. LT forms. Finally, Figure 3 demonstrates that when fractions containing Cx or α_H m.w. LT classes are pooled (horizontal bars, Fig. 2*A*), concentrated (PM10), and rechromatographed on these same columns in low salt buffers, the Cx class elutes as a single peak in its respective m.w. region (Fig. 3*A*). However, in some experiments, rechromatography of the Cx class resulted in the appearance of a small amount of α_H m.w. class activity. Rechromatography of the α_H class in low salt buffer shows a small peak of activity eluting in the void volume, and a single major peak eluting in the α_H m.w. region (Fig. 3*B*). Attempts to rechromatograph α_L m.w. forms have been unsuccessful because the lytic activity associated with the α_L class in these preparations is very unstable.

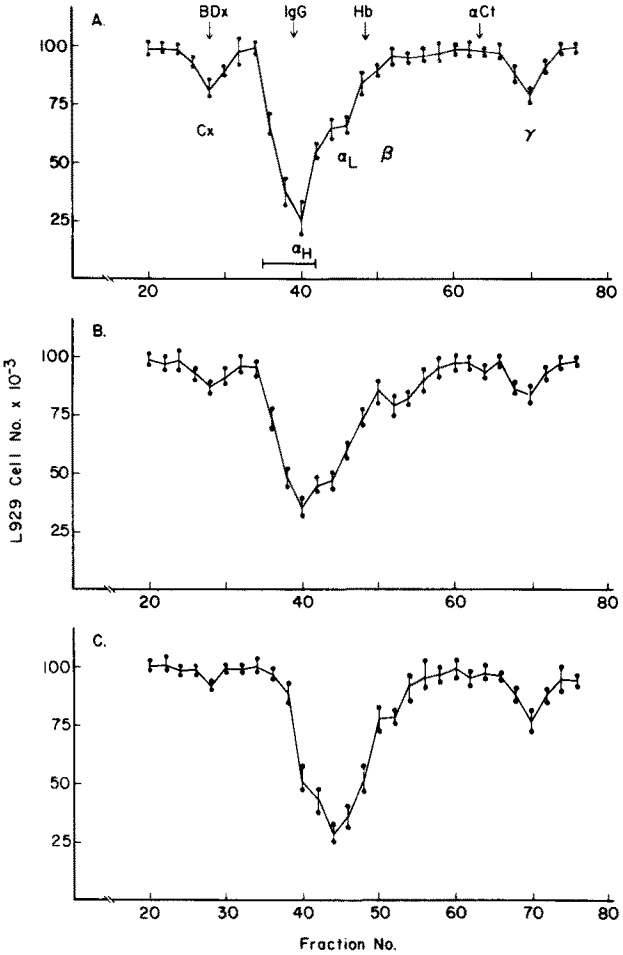

Figure 2. Gel filtration chromatography of LT activity present in supernatants from activated C57BL/6 splenic lymphocytes on Ultrogel AcA 44. *A*, supernatants were collected for nonadherent C57BL/6 splenic lymphocytes activated on PHA/L cell monolayers as described in *Materials and Methods.* These supernatants were concentrated 40 ×, and 2 ml were chromatographed on 2.5- × 90-cm columns containing Ultrogel AcA 44, equilibrated in low salt buffer. Fractions were collected and 100 μl tested for lytic activity on L-929 cells *in vitro*. *B*, same as *A*, except column buffer contained 0.15 M NaCl; *C*, same as *A*, except column buffer contained 0.5 M NaCl.

3. Identification of the m.w. LT classes present in supernatants from activated enriched C57BL/6 T cell populations. The physical characteristics of LT molecules released *in vitro* from activated B6 T lymphocytes were investigated. Nylon wool-purified B6 T cells (95% surface Ig negative) were activated as described in *Methods*, the supernatants were collected, immediately concentrated (40 ×) (PM10), and chromatographed in low salt buffer. LT activity obtained from activated T cells is also heterogeneous. Cx, α_H, α_L, and a small amount of β class activity were observed. Similar profiles were observed in three separate experiments by employing different preparations of T cell supernatants.

4. Resolution of the α_H class of LT molecules into multiple components by ion exchange chromatography and gel electrophoresis (PAGE). Supernatants from B6 splenic lymphocytes were collected, concentrated, and fractionated by gel filtration on AcA 44. Fractions containing α_H LT activity (horizontal bar, Fig. 2*A*, Fx 35–42) were pooled, concentrated, dialyzed against DEAE starting buffer by ultrafiltration, and applied to a 7 ml DEAE column equilibrated in the same buffer. Figure 4*A* shows the results of one of four separate DEAE column separations. Clearly, LT activity present in the α_H class is heterogeneous and can be resolved into at least three components on DEAE. These three components elute at approximately 0.2 M NaCl, 0.3 M NaCl, and the high salt wash. Materials with LT activity within a m.w. class that are resolved by ion exchange columns have been termed subclass components based on terminology describing charge separation of human LT molecules (2). Activity eluting in the nonbinding salt gradient and high salt regions are termed $subclass_1$, $subclass_2$, and $subclass_3$, respectively. Thus, murine α_H m.w. LT molecules can be resolved into a α_{H2a}, α_{H2b}, and α_{H3} activity by their order of elution from DEAE. Furthermore, when identical amounts of these same DEAE fractions were again assayed after storage at 4°C for 48 hr, the lytic activity associated with the α_{H2a} component had decayed and was almost undetectable (Fig. 4*B*). The heterogeneity of α_H LT forms observed on DEAE was also evident when these identical materials were fractionated by PAGE electrophoresis. Figure 5 shows the PAGE profile of α_H LT at pH 9.0. Clearly, these molecules are heterogeneous and are resolved into several peaks that migrate predominantly in the last one-half of the gel.

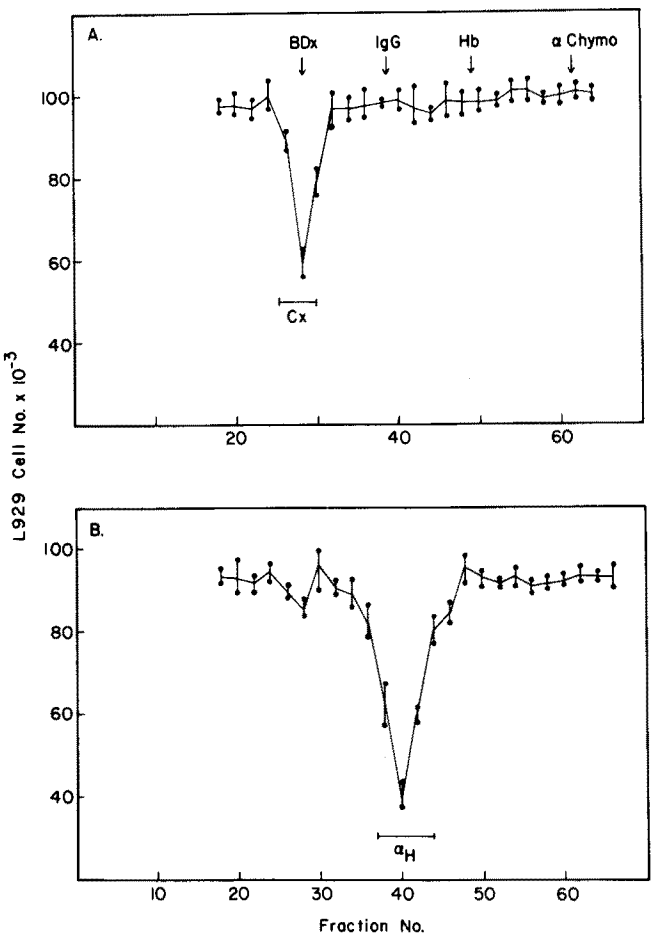

Figure 3. Rechromatography of Cx or α_H m.w. LT classes on Ultrogel AcA 44. *A*, supernatants collected from nonadherent C57BL/6 splenic lymphocytes activated on PHA/L cell monolayers were initially chromatographed on Ultrogel AcA 44 in low salt buffer (Fig. 2*A*). Fractions containing Cx m.w. LT activity (Fx 25–30) from three identical columns were pooled, concentrated to 2 ml (PM10), and rechromatographed on the same Ultrogel AcA 44 column in low salt buffer. Fractions were collected, and 200 μl assayed for toxic activity on L-929 cells *in vitro*. *B*, same as *A*, except fractions containing α_H m.w. LT activity (Fx 36–42, Fig. 2*A*) were pooled, concentrated to 2 ml, and rechromatographed on Ultrogel AcA 44 in low salt buffer.

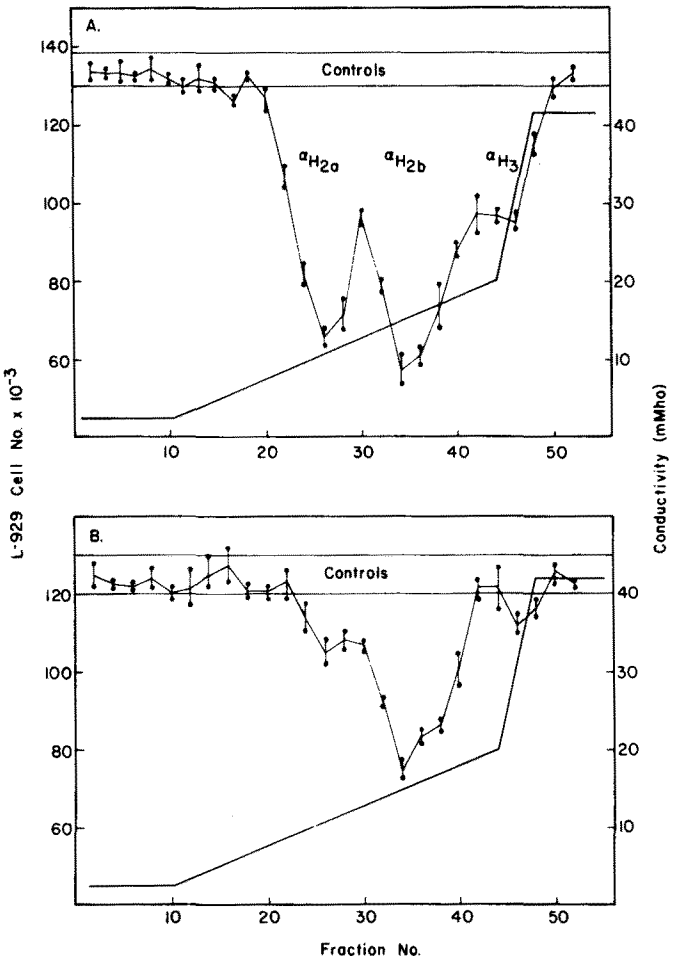

Figure 4. Resolution of α_H m.w. LT class into multiple subclasses by ion exchange chromatography on DEAE. *A*, fractions containing α_H class LT molecules (Fx 36–41, Fig. 2*A*) were pooled from three identical Ultrogel AcA 44 columns, dialyzed against DEAE starting buffer by ultrafiltration (PM10), and 1.5 ml were loaded onto an 8-ml DEAE column equilibrated in the same starting buffer. A linear elution salt gradient (0 to 0.14 M NaCl) in starting buffer was applied to the column followed by a 1 M NaCl high salt wash. Fractions were collected and tested for lytic activity on L-929 cells or conductivity. *B*, same as *A*, except DEAE column fractions were assayed for lytic activity on L-929 cells after storage at 4°C for 24 hr.

5. Development of heterologous rabbit antisera against C57BL/6 whole supernatant (Anti-WS) or isolated α_H (anti-α_H) class LT molecules. During these studies, two types of antisera were produced: 1) anti-whole supernatant produced in protein-free media from stimulated normal C57BL/6-nonadherent lymphocytes; and 2) anti-α_H class sera made against rechromatographed Ultrogel AcA 44 fractions containing the α_H class of murine LT molecules. These antisera were produced in rabbits as described in *Methods*. As can be seen in Figure 6, anti-WS sera can completely neutralize soluble LT mediated lysis of L-929 cells during a 16 hr assay. Inhibition of LT activity was a dose related phenomenon, where 10 μl of sera could inhibit approximately 10 units of LT activity. Antisera made against the α_H form of LT could also neutralize soluble LT activity present in a fresh whole supernatant. However, these sera were weaker, requiring from 7 to 10 times more sera to neutralize the same amount of LT activity. Furthermore, neutralization of lytic activity in whole supernatant by anti-α_H sera was never more than approximately 60 to 80%. That the blocking activity of these anti-LT sera was due to antibody was demonstrated by testing purified IgG fractions. These data are shown in Table I. Also shown in Table I is a composite of many experiments done over a 4-month period, investigating the reactivity of these two sera with various isolated m.w. classes of murine LT molecules present in supernatants from lympho-

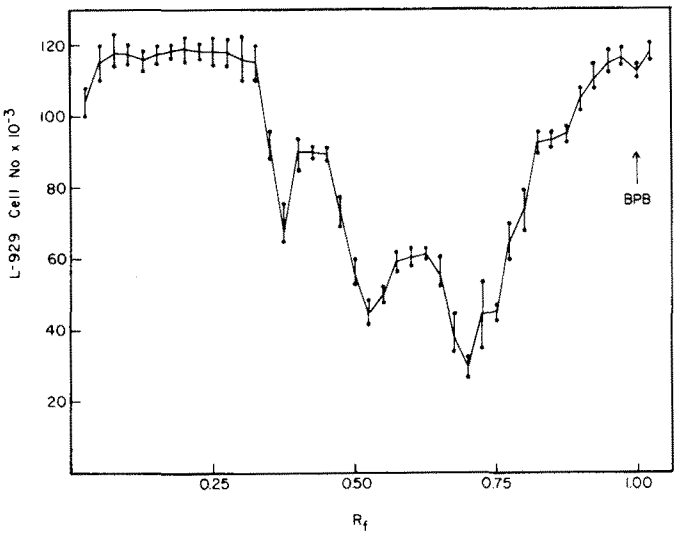

Figure 5. Resolution of α_H class LT molecules into multiple subclasses by PAGE, pH 9. Fractions containing α_H class LT activity were pooled (Fx 36–41, Fig. 2*A*), concentrated to 2 ml (PM10), and 75 μl in 20% sucrose were applied to a 8 cm 7% acrylamide gel containing a 1 cm 3% stacking gel. After electrophoresis for 3 hr at 4°C, the gels were cut into 2-mm sections, and each section was eluted into 0.3 ml RPMI-1640 for 16 hr at 4°C, and 100 μl were tested in duplicate for lytic activity on L-929 cells as described in *Materials and Methods*.

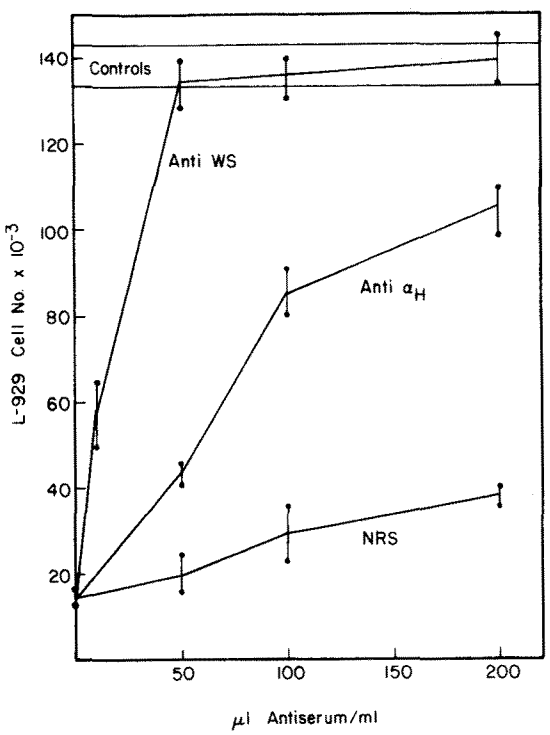

Figure 6. Neutralization of soluble LT activity by heterologous antiwhole supernatant (anti-WS), or anti-α_H class antisera *in vitro*. Various amounts of anti-WS, anti-α_H, or NRS were added to 0.1 ml of C57BL/6 fresh whole supernatants (containing 10 units of toxic activity), and this mixture was added directly to duplicate L-929 cell tube cultures to assay for residual toxic activity during a 14-hr assay at 37°C.

cytes stimulated for 9 hr on PHA/L cell monolayers or for 4 days with PHA alone. We found in these latter supernatants a majority of α_L and β class LT activity, as has been previously reported (11, 12). It is clear anti-WS sera will strongly inhibit LT activity associated with all m.w. LT classes presently identifiable. In addition, anti-α_H sera are also reactive with *all* m.w. LT classes, indicating these various m.w. LT forms are immunologically related. Finally, absorption of these various antisera on L-929 target cells, normal mouse serum, or bovine serum immunoabsorbents did not affect their LT-neutralizing capacity (Table II).

6. Immunologic relationships of murine LT molecules to LT molecules obtained from other animal species. To test whether immunologic relationships existed between C57BL/6 murine LT molecules and LT molecules isolated from a variety of other animal species, we tested these various antisera against LT activity obtained from rat or guinea pig lymphoid cells or human lymphoid cells stimulated as described in *Methods*. The data shown in Table III indicate that although anti-WS and anti-α_H sera can strongly inhibit murine LT activity, they do *not* neutralize LT activity from rat, guinea pig, or human lymphoid cells.

7. Immunologic relationships of C57BL/6 murine LT molecules to the intracellular "toxicity activity" in homogenates of B6 spleen cells in vitro. To test whether LT molecules were immunologically related to nonspecific intracellular cell toxins (i.e., lysozomal enzymes, proteases, etc.), homogenates of unseparated B6 spleen cells were prepared by sonication, as described in *Methods*. These supernatants were tested for toxic activity in the presence of NRS, anti-WS, or anti-α_H sera on L-929 cells *in vitro*. The data are not shown, but anti-WS sera are weakly inhibitory to this toxic activity when high levels of antiserum are employed (100 μl inhibited 21% of this activity). However, neither NRS nor anti-α_H sera inhibited this activity, even at levels of 20% serum (6 and 8% neutralization, respectively).

DISCUSSION

The present studies investigate the physicochemical and immunologic properties of materials with LT activity present in supernatants from murine lymphoid cells activated on lectin-coated allogeneic cell monolayers. These studies clearly dem-

TABLE I

Effect of heterologous anti-WS or anti-α_H LT antisera or IgG fractions on the lytic activity of various MW LT classes present in supernatants from activated murine spleen cells in vitro[a]

Antisera Employed	Method Used to Induce LT	Time of Culture	% Neutralization of LT Class[b]					
			Whole supernatant	Cx	α_H	α_L	β	γ
Anti-WS (25 μl)	PHA/L cell	9 hr	+++	+++	+++	+++	NT	NT
Anti-WS (250 μg IgG)	PHA/L cell	9 hr	+++	+++	+++	NT	NT	NT
Anti-WS (25 μl)	PHA (25 μg/ml)	4 days	+++	NT	NT	+++	+++	+++
Anti-α_H (100 μl)	PHA/L cell	9 hr	++	+++	+++	++	NT	NT
Anti-α_H (1 mg IgG)	PHA/L cell	9 hr	++	++	++	++	NT	NT
Anti-α_H (100 μl)	PHA (25 μg/ml)	4 days	++	NT	NT	++	++	+

[a] To 100 μl of C57BL/6 whole supernatant (containing approximately 10 units LT activity), or 100 μl of Ultrogel fractions containing various MW LT classes (approximately 2 units of LT activity) were added various amounts of anti-WS, anti-α_H, or control NRS or their IgG fractions during an 18-hr incubation with L-929 cells at 37°C. The various MW classes of LT employed in these studies were obtained by Ultrogel chromatography of LT activity present in concentrated supernatants (10 ×) from lymphocytes stimulated on PHA/L cell monolayers for 9 hr or stimulated with 25 μg/ml PHA-P in 5% FCS for 4 days. These latter supernatants were found to have a majority of α_L and β class LT molecules.

[b] Per cent neutralization of LT activity was determined relative to NRS or NRS IgG-treated controls: % Neutralization: +++ = 75 to 100%; ++ = 35 to 75%; + = 10 to 35%; − = 0 to 10%.

TABLE II

Capacity of heterologous rabbit anti-WS, or anti-α_H LT antisera to neutralize soluble LT activity after various absorptions[a]

Serum Employed			Absorbed on	% L-929 Viability	% Neutralization
Anti-WS	Anti-α_H	NRS			
	μl				
		100		18 ± 2[b]	
25				99 ± 2	100
25			L-929	99 ± 2	100
25			NMS beads	97 ± 2	96
25			Bovine serum beads	97 ± 4	96
	100			69 ± 4	62
	100		NMS beads	66 ± 5	60
	100		Bovine serum beads	64 ± 2	56
25				99	Serum alone controls
	100			97	Serum alone controls

[a] To 100 μl of C57BL/6 whole supernatant (10 units of toxic activity) were added 25 or 100 μl of anti-WS or anti-α_H sera which had been absorbed sequentially on: 1) 2×10^8 L-929 cells/ml sera for 2 hr at 4°C; 2) normal C57BL/6 serum or bovine serum covalently linked to Sepharose 4B (2 mg serum protein/ml of beads) 1 ml of antisera/ml beads. LT activity was tested on L-929 cells in the presence or absence of these absorbed sera or control NRS for 15 hr at 37°C, as described in *Materials and Methods*.

[b] Control C57BL/6 LT.

TABLE III

Effect of heterologous rabbit anti-mouse WS or α_H antisera on the LT activity obtained from various animal species in vitro[a]

Animal Species from Which LT is Derived:	Units of LT Activity/ml in Presence of:				
	NRS	Anti-WS	% Neut.	Anti-α_H	% Neut.
Mouse (C57BL/6)	68 ± 6	2	*98*	41 ± 3	*60*
Rat	46 ± 10	42 ± 5	9	48 ± 4	0
Guinea pig	90 ± 5	95 ± 10	0	82 ± 10	6
Human	104 ± 10	97 ± 6	5	99 ± 8	5

[a] Nonadherent lymphoid cells obtained from the spleens of C57BL/6 mice, albino rats, or Hartley guinea pigs (strain 2) were placed on monolayers of PHA-P-coated L-929 fibroblasts in RPMI-1640, 3% FCS for 8 hr at 37°C. LT activity in supernatants of human adenoid lymphocytes was collected after these cells were stimulated with 20 μg/ml PHA-P for 5 days at 37°C. All supernatants were collected and immediately tested for toxic (LT) activity on L-929 cells in the presence of 100 μl of NRS or anti-α_H sera or 30 μl of anti-WS sera during a 15-hr incubation at 37°C.

onstrate that cell-lytic molecules released by nonadherent murine lymphoid cells or purified T cells in this system are heterogeneous and can be physically separated one from another by gel filtration and ion exchange chromatography. This heterogeneity requires the formulation of a standard terminology for adequate discussion of these data. The terminology we have employed in this manuscript is based originally on the nomenclature begun by *Walker et al.* (1) and *Hiserodt et al.* (3), and extended by *Granger et al.* (2), studying human LT molecules. We employ the general term "class" to denote LT molecules that are separable one from another by their m.w. The term "subclass" has been employed to define different LT molecules within an individual m.w. class that have been resolved one from another by ion exchange chromatography or gel electrophoresis.

Cell-lytic molecules released *in vitro* in the present studies by activated normal murine lymphocytes or T cells were heterogeneous and could be separated into at least three major m.w. classes. These three classes were termed Cx (Cx − >200,000 d), α heavy (α_H − between 110 and 150,000 d), and α light (α_L − between 70 and 100,000 d). Furthermore, two minor m.w. classes, β (35 to 50,000 d) and γ (10 to 25,000 d) were also observed in approximately 20% of the column profiles, or in supernatants from 3- to 5-day cultures (3, 21). The percentage of lytic activity found to be associated with these various m.w. LT forms was as follows: Cx (0 to 20%), α_H (40 to 70%), α_L (0 to 25%), β or γ (0 to 10%). Clearly, α_H comprised the majority of LT activity eluting from these columns. It should be emphasized, however, that the percentages of each class observed in a given column profile may not reflect the true percentage of these classes present in the original unseparated supernatant due to differential stabilities of the various classes, and the fact that larger m.w. forms may quickly dissociate into smaller m.w. forms during chromatography. Finally, the manner of lymphocyte activation or type of activating stimulus the lymphocyte encounters at its surface may dictate both the amount and/or m.w. class of molecule(s) released into the supernatant.

The present studies reveal that two forms of α class activity were observed in these early supernatants. Although material similar in size to the α_H form was previously detected in supernatants derived from mouse (12), guinea pig (22), and man (23, 24), its relationship to the α_L and to other m.w. LT forms was unknown. Hiserodt *et al.* (7) have identified that α_H and α_L m.w. forms can be physically dissociated by high ionic strength (0.5 M NaCl) from the human LT complex class. The present studies clearly demonstrate that the α_H and α_L m.w. forms are physically related. α_H could be dissociated into α_L by chromatography in high ionic strength (0.5 M NaCl) buffers, indicating these subunit forms are associated by only weak molecular forces. Furthermore, as will be discussed in a subsequent paragraph, it was found that antisera directed against the α_H m.w. LT form could neutralize the lytic activity associated with α_L. Thus, α_H and α_L m.w. LT forms appear to be related both physically and immunologically. The precise molecular relationships of α_H to the other m.w. LT classes will be thoroughly discussed in the third manuscript in this series.

The present studies indicate that the amount of each class detectable in a given supernatant can vary. We feel that variation can be influenced by several parameters. When 8-hr supernatants are stored at 4°C, all m.w. murine LT classes lose activity rapidly and are completely undetectable after 3 or 4 days. This is a serious problem since extensive biochemical characterizations of these molecules will be extremely difficult without an effective method for stabilizing these materials. Preliminary experiments suggest that the lytic activity associated with these molecules may be at least partially stabilized by adjusting the pH, but was unaffected by the addition of various protein carriers.

The murine α_H LT molecules appear to be heterogeneous with respect to charge and can thus be resolved into several subclasses. The same nomenclature employed for human α_L class charge heterogeneity has been applied in referring to these molecules (2). Materials present in the α_H m.w. class were resolved into at least two subclasses by ion exchange chromatography. Whereas α_{H2a} and α_{H2b} represent the predominant molecular species of α_H, α_{H3} accounts for only a small amount (between 5 and 15%) of the total α_H class activity. These findings are similar to those reported for human α_L class charge heterogeneity, in which fractionation on DEAE resolves three separable α subclasses (2). However, these molecules differ from those of human in that they are more highly charged. It should also be emphasized that the α_H form can dissociate in high ionic strength buffers into the α_L form. Therefore, some of this heterogeneity may actually represent α_H that has dissociated into α_L. We were not able to determine if this was the case, for these molecules lost activity and could not be rechromatographed over Ultrogel to determine their m.w. We feel the present data strongly support the concept that, like human LT molecules, the various forms of murine LT molecules are related and form a subunit system.

Other investigators have examined the physicochemical properties of cell-lytic molecules, termed LT, released *in vitro* by lectin-activated murine lymphoid cells. Studies by Kolb and Granger (11) revealed murine LT molecules present in 2 to 3 day cultures of lymphocytes activated on PHA-coated L-929 monolayers eluted in a broad m.w. range between 80 and 150,000 d by molecular sieving on Sephadex G-150. A more recent study by Trivers *et al.* (12) demonstrated that LT activity in 3-day PHA-stimulated cultures eluted from Sephadex G-200 in the 45,000 d region. However, other minor peaks were noted, but not emphasized, eluting in the void volume (>200,000 d) and with the IgG marker (150,000 d). Although these reported differences in observed m.w. values of materials with LT activity are difficult to compare, Trivers suggested that these various m.w. LT forms may be related and represent subunit structures. This concept was supported in the present study by the following considerations: a) Cx and α_H m.w. forms were shown to be physically related to the α_L m.w. form(s), for Cx and α_H could be dissociated into the α_L form(s) under

conditions of high ionic strength: b) anti-α_H sera could neutralize the lytic activity associated with *all* m.w. LT forms detected in these supernatants, strong evidence that these various classes of LT are immunologically related. In addition, when we tested fractionated supernatants collected from 4-day lectin-activated murine lymphoid cells, we found a majority of the smaller m.w. forms, (α_L, β, and γ) similar to the findings of previous investigators (11, 12). These various m.w. classes were also strongly inhibited by anti-WS *and* anti-α_H sera. Thus, the smaller m.w. forms of murine LT seen by other investigators are immunologically related to the higher m.w. forms observed in the present studies. Therefore, we feel the differences in reported m.w. values for murine LT forms studied in different laboratories do not reflect detection of different molecules but instead various m.w. forms of the same LT system of cell toxins, since the conditions under which these molecules are isolated (i.e., pH, salt, time of collection, culture conditions) have dramatic effects on the m.w. form(s). Furthermore, the high levels of cytolytic activity detected in these early supernatants may not represent *more* LT molecules, but higher m.w., more lytically active forms, which may decay or dissociate into smaller m.w., less active form(s) with time.

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