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The Human LT System[1]

I. Physical-Chemical Heterogeneity of LT Molecules Released by Mitogen Activated Human Lymphocytes *in Vitro*

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Molecules with LT activity which can be identified in supernatants from PHA stimulated human lymphocytes by lysis of murine L-929 cell *in vitro* are heterogeneous. They can be separated by MW on sephadex or ultrogel columns into four separate classes: (a) complex (>200,000 daltons); (b) α (70–90,000 d); (c) β (25–50,000 d); and (d) γ (10–20,000 d). The amount of activity in a supernatant due to each class varies but is approximately: *Cx*—5 to 20%, α—40 to 60%, β—20 to 40%, and γ—0 to 10%. These classes differ one from another in their stability and kinetics of appearance in culture. Furthermore, they may aggregate together with the complex class under conditions of low ionic strength. Each class, except γ, can be further separated into subclasses by ion exchange chromatography and polyacrylamide gel electrophoresis. Alpha class can be separated on DEAE into the three subclasses. termed, α_1 (rf 0.25), α_2 (rf 0.37), and α_3 (rf 0.50). Alpha$_2$ subclasses can be further separated on phosphocellulose at pH 6.6 into α_{2a} (rf 0.37) and α_{2b} (rf 0.30). However, α_2 contains additional subclasses, which were resolved on PC columns at pH 5.5. Beta class activity can be resolved by DEAE and PAGE into two subclasses, termed β_1 (rf 0.28) and β_2 (rf 0.49). Gamma class activity was not studied, because of its instability. The complex class of LT activity is a macromolecular aggregate greater than 200,000 daltons which appears to contain smaller MW LT class(es). This study demonstrates that materials with LT activity in supernatants from PHA activated human lymphocytes *in vitro* are very heterogeneous.

INTRODUCTION

Lymphocytes from experimental animals and man, when activated by co-culture with antigens or mitogens *in vitro,* release a complex family of soluble effector molecules, termed lymphokines, which have a wide range of biologic activities (1). Lymphotoxins (LT), substances which are cytotoxic for cells *in vitro,* are traditionally classed as lymphokines (2). These material(s) have been proposed as soluble lytic effectors in tissue destructive cell-mediated immune reactions. However, recent studies indicate they can also exist in a membrane associated form (3, 4). Lymphotoxin(s) obtained from lymphocyte cultures derived from man and

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experimental animals are relatively nonspecific, however, there is a wide range of cell sensitivity to their lytic effects *in vitro* (2, 5–7). Physical-chemical studies of materials with LT activity has been difficult, for while they have high biologic activity, they are present in lymphocyte supernatants in very small amounts. The situation is also complicated by the finding that LT activity can be detected in supernatants of lymphoid cells when stimulated by diverse and unrelated agents, i.e., soluble antigens, mitogens, and allogeneic cells (2, 8). Moreover, certain continuous human lymphoid cell lines spontaneously release material(s) with "LT activity" (9, 10). Lymphotoxins have been shown to have a role as lytic effectors in certain forms of tissue destructive CMI reactions *in vitro* (11, 12). Because of these findings, there is a definite need for characterization of these molecules to help define their precise role in cell lytic CMI reactions.

Lymphotoxins released *in vitro* by activated lymphoid cells from the rat, mouse, and guinea pig have been subjected to physical-chemical studies. These material(s) appear to be proteins which are heterogeneous with respect to molecular weight. Materials with LT activity released by mitogen activated murine spleen cells *in vitro* were reported in one study to be 35 to 45,000 MW (13), and in another, 80 to 150,000 MW (14). Lymphotoxin(s) released by mitogen stimulated rat lymphoid cells *in vitro* were reported to be 70 to 90,000 MW, with some indication that there may be small amounts of material 35 to 45,000 MW (15). In contrast, guinea pig lymphotoxin released by antigen stimulated immune lymphoid cells in one study was 45,000 MW, with no evidence of larger molecules (16).

Materials with LT activity released by activated human lymphocytes or continuous lymphoid cell lines *in vitro* have been the most studied. Initial physical studies of LT activity in supernatants from mitogen activated normal human lymphocytes obtained from tonsils or adenoids *in vitro* revealed they possessed the following properties: 70 to 90,000 MW, stable to heating at 56°C for 15 to 30 min, protein in nature, and migrate in electrophoresis in the range of the fast gamma or slow beta globulins (17, 18). Material(s) with similar lytic activity, heat sensitivity, and molecular weight were reported to be spontaneously released by a number of continuous human lymphoid cell lines *in vitro* (9, 10). Walker, *et al.*, were the first to report LT-like material(s) in supernatants of mitogen stimulated lymphocyte cultures that were in the 45,000 MW range. They termed the 70 to 90,000 MW materials "alpha", and these latter as "beta" lymphotoxins (19). Similar and concurrent studies by Hiserodt and Granger confirmed this report (20). In a subsequent publication, Lee and Lucas were able to further separate the α lymphotoxins obtained from PHA stimulated human tonsil cells into three subclasses by ion exchange chromatography and polyacrylamide gel electrophoresis (21). Shortly thereafter, Hiserodt *et al.*, were able to fractionate the beta group into two subclasses, β_1 and β_2 by ion exchange and polyacrylamide gel electrophoresis (22).

The present report is the first of a series of studies designed to examine the physical and immunologic characteristics and relationships of human LT molecules in supernatants derived from mitogen stimulated human lymphocytes *in vitro*.

MATERIALS AND METHODS

Target Cell and Culture Media

Target cells were obtained from stock cultures of mouse L-929 fibroblasts maintained in RPMI 1640 (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 3% heat-inactivated (56°C, 1 hr) fetal calf serum (Grand Island

Biological Co., Grand Island, N.Y.) 100 U/ml penicillin, and 100 μg/ml streptomycin (RPMI-S). These cells were grown at 37°C in 32-oz prescription bottles in 95% air, 5% CO_2, and passed biweekly.

Production of Human LT

The details of these methods have been described previously (23). Briefly, suspensions of human lymphocytes were obtained from tonsils or adenoids of patients 2 and 4 hr after surgical removal. Cultures were established at 4×10^6 viable cells/ml (viability routinely 75 to 85%) in 32-oz prescription bottles in RPMI 1640, supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin, 5×10^{-5} *M* mercaptoethanol, and 20 μg/ml of a heat stable bovine serum fraction. Activation of the lymphocytes was affected with phytohemagglutinin-P (PHA-P, Difco, Detroit, Michigan) at 20 μg/ml. The cells were then maintained for 5 days at 37°C in 95% air, 5% CO_2. Supernatants were collected, cleared of cells by centrifugation, filtered through glass fiber filters, and stored at −20°C. The supernatant from the activated human lymphoid cells (SAL) was pooled into 10 L lots, concentrated 50-fold by passage through a Bio-Rad Hollow Fiber Concentrator with a pore size retaining molecules greater than 30,000 MW, divided into aliquots, and stored at −20°C or −70°C until used. These studies have employed seven separate pools. Concentration of SAL has also been performed employing Amicon PM10 membranes in order to retain molecules having MW greater than 10,000 d.

Preparation of ^{14}C Amino Acid Labeled LT

Supernatants containing radiolabeled effector molecules were produced by two separate methods: (a) lymphoid cells were established in MEM lacking either phenylalanine or leucine, but supplemented with 5 μCi/ml of ^{14}C phenylalanine (305 mC/m*M*) or ^{14}C leucine (368 mC/m*M*), respectively. In addition, each culture received 10% of the normal 1 × MEM level of nonradioactive phenylalanine or leucine and stored exactly as described above; (b) cells were established in MEM containing 5% FCS for 24 to 72 hr, then washed and shifted into labeling medium, as described above for an additional 24 to 48 hr. The SAL was collected and stored at −20°C.

Lymphotoxin Assay

Two types of assay were employed, one which determines quantitatively the amount of LT activity present in a given supernatant, and a second which only indicates qualitatively its presence or absence. These techniques have been described in detail previously (24). Briefly, monolayers of an LT sensitive strain of mouse L-929 fibroblast cells were established in 16 × 125 mm glass screw-capped culture tubes at a density of 100,000 cells/ml in RPMI-S, containing 0.5 μg/ml mitomycin C (Sigma Chemical Co., St. Louis, Mo.) and incubated in 95% air, 5% CO_2 for 24 hr. After this 24 hr incubation at 37°C, the medium was discarded, and to determine the amount of LT present in a sample, serial dilutions of LT-containing or control media in RPMI-S were added to duplicate cultures, and they were incubated for 24 hr at 37°C. The number of remaining viable adherent cells were determined by trypsinizing the cells and passing them through a Model F Coulter Counter. One unit of LT activity is defined as that which will destroy 50% of

the target cells. The reciprocal of the LT dilution killing 50% of the target cells in 18 to 24 hr yields the number of units of LT/ml in the original undiluted supernatant. To determine the presence of LT in a fraction, an aliquot of the fraction (0.01 to 0.2 ml) was added to 1.0 ml tube cultures established as described above. The number of viable cells remaining was determined also as described above. The percent viability was calculated by the following formula:

$$\frac{\text{cell No. (test)}}{\text{cell No. (control)}} \times 100.$$

Physical-Chemical Separation of LT

Certain of the methods employed for fractionation of LT activity in supernatants from activated human lymphocytes have been previously published (25). All procedures were performed at 4°C.

A. *Molecular sieving.* Degassed Ultrogel AcA-44 (LKB, Upsala, Sweden), or Sephadex G-150 (Pharmacia, Upsala, Sweden) were poured to a bed height of 95 to 100 cm in 2.5 × 120 cm siliclad coated columns, equilibrated in 10 m*M* 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 [2×10^6 daltons (d)], bovine serum albumin (BSA-68,000 d), ovalbumin (OA-44,000 d), and myoglobin (17,500 d), applied in a total volume of 1.5 to 2.0 ml. Six ml fractions were collected at a flow rate of 24 ml/hr, and monitored for their absorbence at 280 nm on a Gilson Model 2000 Spectrophotometer (Middleton, Wis.). One and one-half to 2.0 ml 50 × SAL samples containing from 5000 to 20,000 units of LT activity were chromatographed over these columns, and 0.05 to 0.2 ml samples of the eluted fractions were assayed for LT toxic activity. Larger preparative columns, 5 × 100 cm were also employed in these studies. Rechromatography of the various LT classes was performed in a fashion identical to the original separation procedures. Fractions were stored at 4°C.

B. *DEAE-cellulose chromatography.* DEAE (DEAE 11, Whatman) was washed, degassed, and poured to a bed height of 20 cm in a 2.5 × 34 cm siliclad-coated glass column and equilibrated in 10 m*M* Tris-HCl, pH 8.0, 0.1 m*M* EDTA. Before chromatography on DEAE, samples were routinely treated with DNAse. Samples were dialyzed against PBS for 24 hr and subjected to DNAse treatment (80 units Pancreatic DNAse A/ml) for 2 hr at room temperature in the presence of 10 m*M* Mg Cl_2, 10 m*M* $CaCl_2$. Rechromatographed LT fractions from several molecular sieving columns were pooled, tested for LT activity, and concentrated by pressure ultrafiltration to 2.0 ml on Amicon PM-10 membranes. They were dialyzed overnight against 1,000 volumes of 10 m*M* Tris, pH 8.0, 0.1 m*M* EDTA. Each fraction tube received approximately 200 μg BS as a carrier before the fractions were collected from these columns. Protein was eluted with a linear 500 to 600 ml gradient from 0 to 0.3 *M* NaCl, in 10 m*M* Tris, pH 8.0, 0.1 m*M* EDTA, followed by 50 ml of 1.0 *M* NaCl in the same buffer. Six ml fractions were collected at a 30 ml/hr flow rate and tested for conductivity, and 0.01 to 0.05 ml tested for LT activity.

C. *Phosphocellulose (PC)* (Bio Rad, Los Angeles, California). A 2.5 × 10 cm column of PC was poured in a siliclad coated glass column. The column was

equilibrated in 10 m*M* potassium phosphate, 0.1 m*M* EDTA (pH 5.5 or 6.6) (starting PC buffer). Fractions from DEAE columns were pooled and concentrated to 1 to 2 ml by pressure ultrafiltration on PM-10 membranes. The samples were then dialyzed against 200 to 1000 volumes of starting PC buffer. Samples were eluted with a 500 ml linear gradient from 0 to 0.3 *M* NaCl, followed by 50 ml of 1.0 *M* NaCl in the same buffer. Six ml fractions were collected at a flow rate of 20 to 40 ml/hr, tested for their conductivity, and 0.01 to 0.05 ml tested for LT activity.

D. *Polyacrylamide disc gel electrophoresis (PAGE).* PAGE was performed by the method of Davis (26). A 100 to 200 μl sample 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 m*M* 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 marker. The gel samples routinely contained from 20 to 100 units of LT activity. PAGE performed on radioactive samples were cut, solublized in 3% protosol, and suspended in Omnifluor-toluene, and radioactivity determined in a Beckman LS-233 Scintillation Counter.

RESULTS

Definition of the Various MW Classes of Human LT in PHA Stimulated SAL by Gel Filtration Chromatography

Cytotoxic activity associated with these supernatants were concentrated and subjected to gel filtration chromatography. Shown in Fig. 1A is an elution profile in PBS buffer, which is typical for these SAL concentrates on Sephadex G-150. The elution profile of the various MW markers in PBS buffer, blue dextran, BSA, oval, and Myo is also indicated. It is clear that cytolytic activity can be resolved into four molecular weight classes we have called complex [> 200,000 daltons (d)], alpha (70–90,000 d), beta (25–50,000 d) and gamma (10 to 20,000 d). Similar profiles were obtained when 50 × SAL concentrates were chromatographed on AcA-44 columns (Sepharose-acrylamide beads) under identical conditions as shown in Fig. 1B. These figures do not represent individual column profiles, but are general profiles averaged from 30 to 40 different runs employing several different pools of concentrated SAL. Because AcA-44 columns do not pack, we discontinued use of the sephadex columns. While these LT profiles were highly reproducible, we tested a number of AcA-44 columns equilibrated in 0.01 *M* phosphate, pH 7.2, in no salt, and observed the profile(s), shown in Fig. 1C. It is clear these materials may aggregate in conditions of low ionic strength. It is also possible that LT complex is more stable under conditions of low ionic strength. However, we have stored these same complex LT fractions in either low salt or PBS conditions for up to 2 months at 4°C, with little or no loss of toxic activity. Larger AcA-44 columns were also employed (4.5 × 90 cm) to permit separation of more material. The fractions containing the molecular weight classes complex, α-, β-, and γ-LT activity were routinely pooled as shown in Fig. 1C, and concentrated by ultrafiltration through a PM10 Amicon membrane to 2 ml and rechro-

matographed on identical Ultrogel AcA-44 columns in no salt or 0.15 *M* NaCl in phosphate buffer. As shown in Fig. 2A, B, and C, each class contains a small percent of other MW classes. However, they can be further resolved to remove the other classes by this procedure. The elution profiles were quite similar, whether the columns were equilibrated with 0.15 *M* NaCl or salt free. The LT classes β

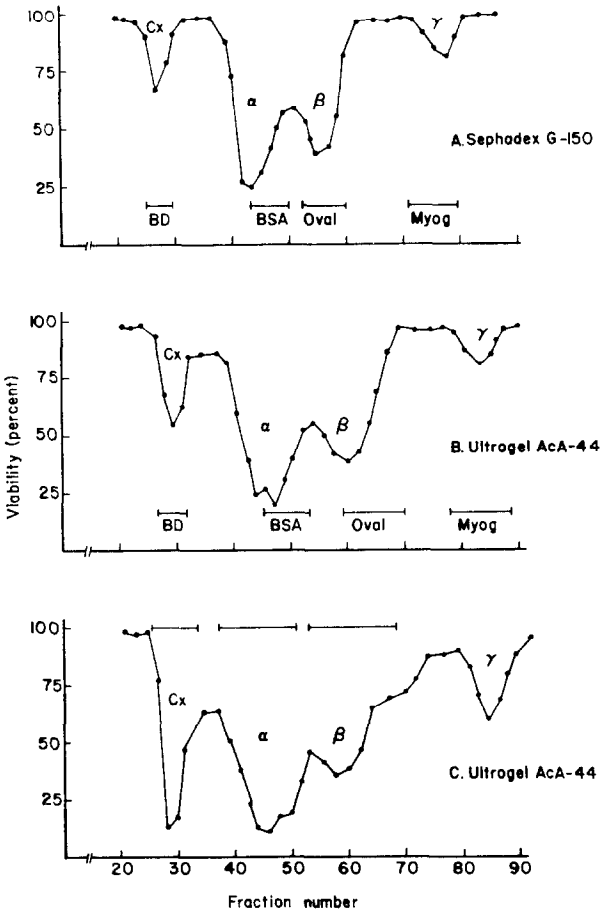

FIG. 1. Chromatography of 5-day supernatant from PHA activated human lymphocytes (SAL) on AcA 44 and Sephadex G-150 columns. The elution profile of various molecular weight markers is indicated by horizontal bars. They are: Blue Dextran (BD)—> 2×10^6 MW, Bovine Serum Albumin (BSA)—67,000 MW, Ovalbumin (Oval)—45,000 MW, and Myoglobin (Myo)—18,000 MW. A. Sephadex G-150. Columns were equilibrated and eluted with PBS containing 10^{-4} *M* EDTA. A 1.5 ml sample of 50 × SAL concentrate was chromatographed over a 90 cm gel bed in a 2.5 × 120 cm column. Six milliliter fractions were collected at 24 ml/hr and 100 μl samples were tested from every other fraction for LT activity, as described in *Materials and Methods*. Toxic activity of each sample is expressed as % viability. B. Ultrogel AcA 44. Columns were equilibrated and eluted with PBS containing 10^{-4} *M* EDTA. A 1.5 ml 50 × SAL concentrate was chromatographed over a 90 cm gel bed in a 2.5 × 120 cm column. Samples were collected, tested, and results expressed in a manner identical to those shown in Fig. 1A. C. Ultrogel AcA 44. Columns were equilibrated and eluted with 0.01 *M* phosphate containing 10^{-4} *M* EDTA and no salt. A 1.5 ml 50 × SAL concentrate was chromatographed and tested for LT activity, as described in Fig. 1A. The horizontal bars represent fractions collected for pools of complex, α, and β-LT classes.

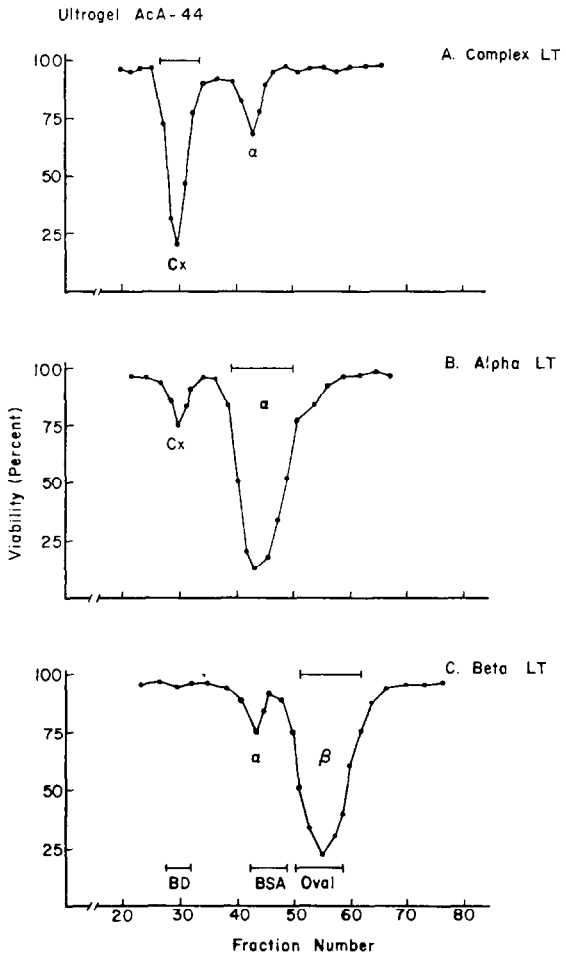

FIG. 2. Rechromatography of various LT classes over ultrogel AcA 44 columns. Fractions containing the various LT classes, shown in Fig. 1, were rechromatographed over 2.5 × 90 cm ultrogel columns to remove other class contaminants. 1.5 to 2.0 ml concentrates, obtained from 1 to 3 preparative ultrogel column runs, were applied to a second column. Collection of fractins and LT testing were as described in Fig. 1. The horizontal bars indicate fractions pooled for subsequent fractionation on DEAE cellulose. Buffer employed for rechromatography was identical to that used in Fig. 1C. A. Rechromatography of complex. B. Rechromatography of α class. C. Rechromatography of β class.

and γ were stable when present in the concentrate (50 ×) frozen for up to one and one-half years. However, upon separation, they rapidly lost activity, whereas the complex and α classes were very stable. While the amounts of activity in each peak vary, depending upon the lot of supernatant, the amount of activity applied to the column, and the amount tested, the same general profiles have been observed in approximately 30 to 40 separate column runs. The amount of activity in a given pool can vary, as previously described (27). To determine the relative amounts of activity in each class and to minimize loss during concentration, we have also employed Amicon PM10 membranes, which retain molecules having MW > 10,000 d. Under our culture conditions, the amount of activity due to individual classes are approximately: complex—5 to 20%, α—40 to 60%, β—20 to 40%,

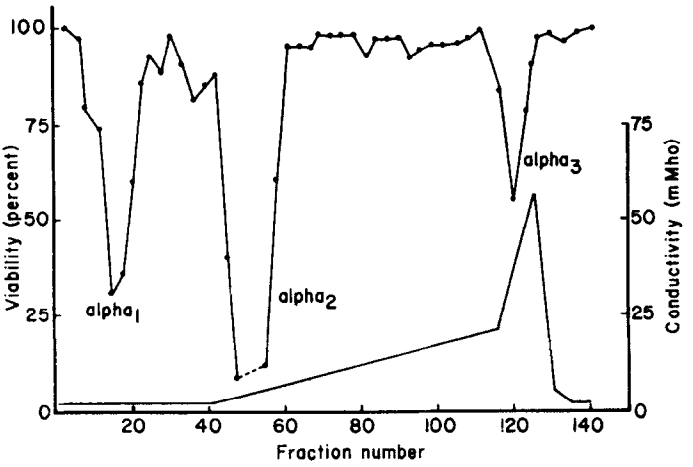

FIG. 3. DEAE cellulose chromatography of α-LT class. Ultrogel rechromatographed α-LT fractions were concentrated, treated with DNAse, and dialyzed against DEAE starting buffer. The sample was applied to the column and eluted with a 500 to 600 ml, 0 to 0.30 *M* NaCl gradient, in 0.01 *M* Tris, pH 8.0. Alternate fractions were tested for conductivity, and 25 to 100 μl were routinely tested for LT activity.

γ—0 to 10% of the total activity. The amount of activity in a given LT pool varies from 200 to 400 units/ml. The total activities recovered range from 75 to 90%.

Resolution of the α-LT Class into Multiple Components (Subclasses) By Ion Exchange Chromatography and PAGE

The fractions containing α-LT activity, after rechromatography on Ultrogel AcA-44, were pooled, concentrated, DNAse treated, dialyzed against starting no salt buffer for 12 to 24 hr, and then chromatographed on a DEAE-cellulose column pre-equilibrated in the same buffer. The total activities from several ultrogel columns were usually pooled together. The elution profile of α class LT on an individual DEAE column is shown in Fig. 3. The first region of activity elutes off the column in 0.0 *M* NaCl. The second appears at a salt concentration of approximately 0.05 *M* NaCl, and the third elutes off with the 1 *M* NaCl wash. These activities have been termed α-LT_1, α-LT_2, and α-LT_3 subclasses, respectively. Over 70% of the total LT activity recovered elutes with the α_2 subclass. While this figure represents an individual elution profile, similar results have been obtained on numerous separations. However, as described by Lee and Lucas, the elution profiles are much broader without DNAse treatment (21). Fractions containing each activity were pooled, concentrated, and subjected to PAGE. Additional studies were performed with radiolabeled supernatants. Typical profiles of these fractions on PAGE are shown in Fig. 4A and B. While α_1-LT toxic activity and ^{14}C counts were confined in a single defined region (Rf 0.25 ± 0.02), α_2 toxic activity was diffuse and widespread (Rf 0.37 ± 0.07). We loaded approximately 40 to 100 units of LT activity/gel. $Alpha_3$ activity has not been intensively studied, but initial PAGE gels indicate it migrates as a broad single peak with an Rf of about 0.5. Because of the diffuse profile of α_2 on PAGE, we further separated these materials on a different type of ion exchange column. DEAE fractions containing α_2 activity were pooled, concentrated and dialyzed against starting PC buffer, and

chromatographed over PC. The resolution of additional α_2 subclasses on PC at pH 6.6 is shown in Fig. 5. Clearly, two peaks of activity, termed α_{2a} and α_{2b}, are resolved. The amount of activity associated with each peak varies, but the predominant activity elutes with the α_{2a} molecules. Fractions containing these subclasses were concentrated and subjected to PAGE, as shown in Fig. 4C and D. $Alpha_{2a}$ migrates as a relatively narrow band with a shoulder and sharp peak with an Rf 0.37 ± 0.07, and α_{2b} migrates as a sharp peak with an Rf of 0.30 ± 0.02. Additional PC columns were run with α_2-LT subclass at pH 5.5 employing the same salt gradient. These data are shown in Fig. 6. It is clear that at a lower pH, materials that had previously eluted with the α_{2a} subclass were now retained by the column. Thus, the α_{2a} subclass shown in Fig. 5 contains additional but separable components.

Resolution of β-LT Class Activity into Multiple Subclasses by Ion Exchange and PAGE

Studies on β class LT activities have been much more difficult, because of the inherent instability of these materials (19). The present studies were possible, because they are much more stable in MEM + BS than serum-containing medium

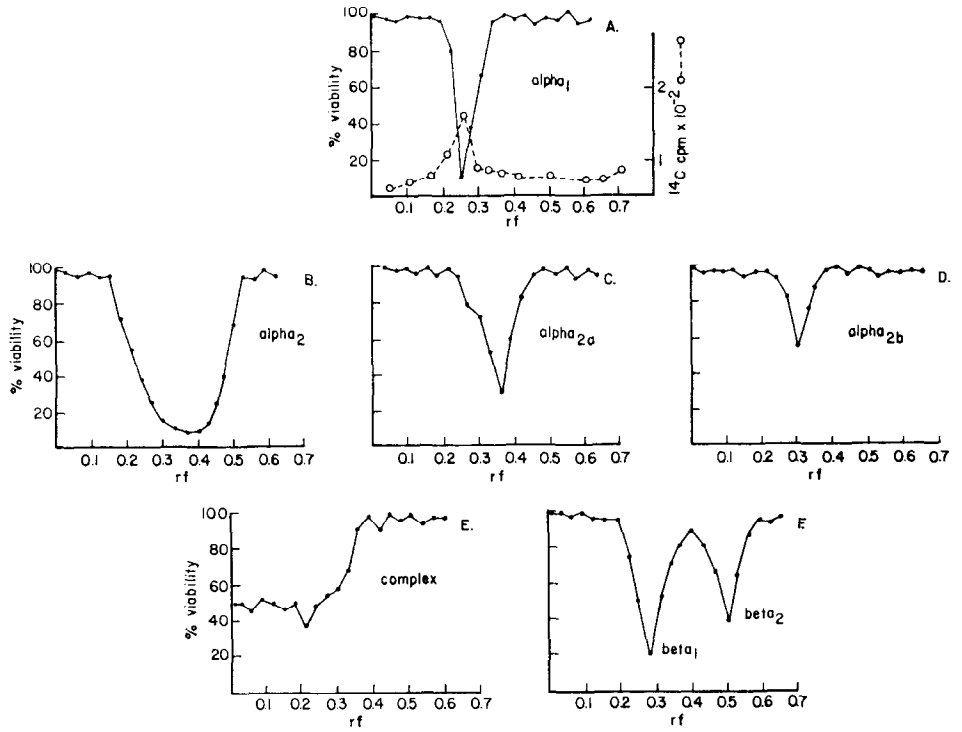

FIG. 4. Polyacrylamide gel electrophoresis of samples containing human LT activity. Fractions (100 μl) of the various human LT subclasses, obtained at various stages of the separation procedures, were subjected to PAGE electrophoresis, as described in *Methods*. The samples applied to gels were obtained from various stages of the separation procedure outlined in the text. The gels were sliced, LT eluted, and tested for toxicity or radioactivity as described in *Methods*. A. $Alpha_1$-LT activity and ^{14}C AA labeled protein. B. $Alpha_2$. C. $Alpha_{2a}$. D. $Alpha_{2b}$. E. Complex. F. $Beta_1$ and $Beta_2$.

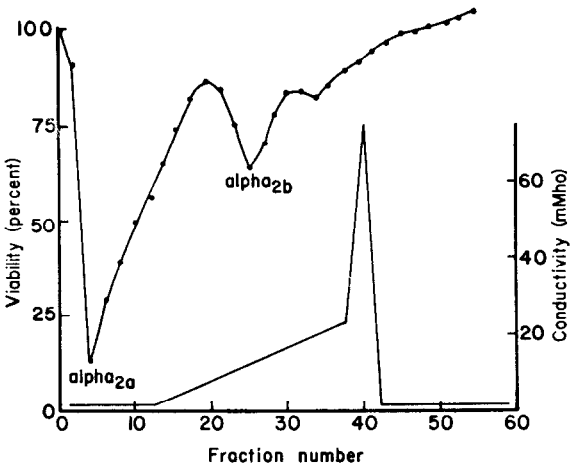

FIG. 5. Separation of α_2 Subclass into Multiple Components by Chromatography on Phosphocellulose at pH 6.6 Fractions containing α_2 LT subclass activity, obtained after DEAE-cellulose chromatography, were pooled, concentrated, dialyzed against starting PC buffer at pH 6.6, and applied to a PC column. Fractions were eluted with a 500 ml, 0 to 0.30 *M* NaCl gradient in the same buffers and tested for conductivity and LT activity.

(28). However separations were conducted in such a fashion so that there was minimum delay between various fractionation procedures.

Fractions containing β-LT activity, after rechromatography on Ultrogel AcA-44 were pooled, concentrated, dialyzed by ultrafiltration against starting buffer and 4.0 ml were layered on a 2.5 × 20 cm DEAE-cellulose column pre-equilibrated in the same buffer. The column was then washed with 100 ml of starting buffer and a 400 ml NaCl linear gradient (0.0 *M* to 0.3 *M* NaCl) was applied. Fractions were collected (5.0 ml) and tested for LT activity. The results of such a separation is shown in Fig. 7. Peak 1 elutes immediately off the column with 0.0 *M* NaCl at pH 8, a second peak elutes off at a salt concentration of 0.12 *M*. These subclass activities have been termed β-LT_1 and β-LT_2, respectively. In addition, the rechromatographed AcA-44 fractions containing peak β-LT activity were subjected to PAGE. A 100 μl sample containing approximately 50 units of activity was applied to PAGE gel and subjected to electrophoresis. The results shown in Fig. 4F are typical of several experiments and indicate that β class LT can also be separated into two distinct peaks of activity, β_1 and β_2, with rf values of 0.28 and 0.49, respectively, relative to the migration of the bromphenol blue marker. Additional studies have revealed β_1 is highly unstable to heating at 56° and storage, while β_2 is stable under these conditions (22).

Examination of Complex and γ-LT Classes by Ion Exchange Chromatography and PAGE

Ultrogel rechromatographed fractions containing complex LT activities were concentrated and subjected to DEAE chromatography under the standard conditions described in *Materials and Methods*. While the data are not shown, the material is heterogeneous, for three broad peaks of activity were detected in positions roughly similar to those observed for the α subclasses 1, 2, and 3. In addition, these same Ultrogel samples were electrophoresed on standard gels, as shown in

Fig. 4E, complex activity migrates with a broad profile over the first 1/3 of the gel. In other gels, additional peaks of activity appeared with rf values from 0.24 to 0.4. Clearly, this material is quite heterogeneous. More detailed studies of complex are reported in another manuscript of this series (29). Attempts to study the γ-LT class of activity have to date met with failure. The material appears, in our conditions, to be highly unstable.

DISCUSSION

Clearly, there are multiple molecules with LT activity released by lectin activated human lymphocytes *in vitro,* and therefore, a need for standard terminology when dealing with these materials. The terminology employed in this manuscript is based originally on the nomenclature begun in the studies of Walker *et al.* (19). We have employed the general term "class" to denote those members of the LT system that are separable by their molecular weight. The term "subclass" has been employed to define different molecules with LT activity which can be further resolved one from another by ion exchange chromatography or electrophoretic techniques from within an individual class. Human LT molecules released by lectin activated human lymphocytes *in vitro* are separable into four MW classes, complex, which is greater than 200,000 daltons (d), α, between 70,000 and 90,000 d; β, 25,000 to 50,000 d; and γ, 10,000 to 20,000 d. These MW classes are similar when chromatographed on two different types of molecular sieving columns, Sephadex G-150 or Ultrogel (Sepharose-polyacrylamide AcA-44). It is important to recognize that these materials may form complexes with one another in buffers of low ionic strength on these columns during primary separation of whole supernatants or concentrates. Thus, unless attention is given to employing the proper

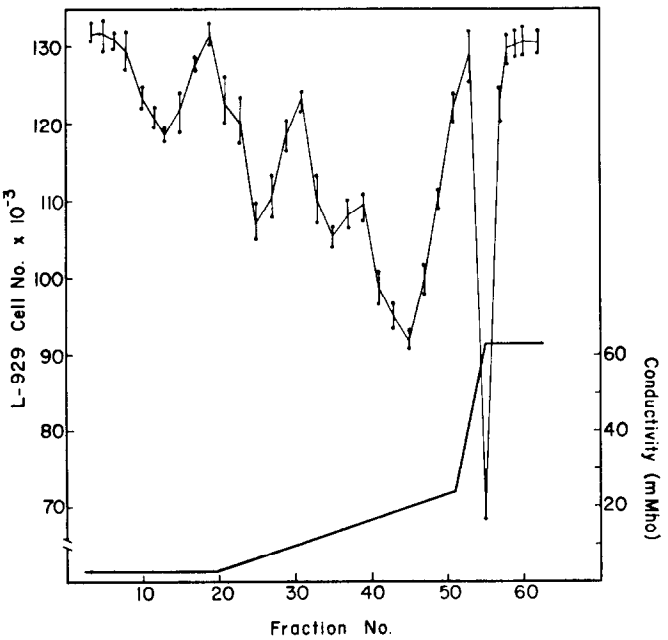

FIG. 6. Separation of α_2 subclass on PC columns at pH 5.5. Samples containing α_2 LT activity were obtained, separated and tested as described in Fig. 5.

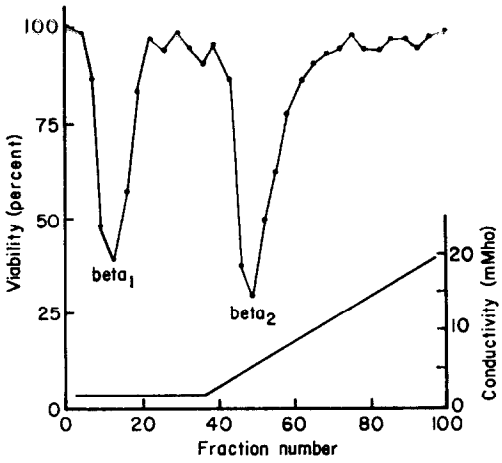

FIG. 7. Separation of β-LT class activity by DEAE cellulose chromatography. Ultrogel rechromatographed β-LT fractions were pooled, concentrated, and chromatographed over DEAE with 0 to 0.35 *M* NaCl gradient, in 0.01 *M* Tris, pH 8, as described in *Methods*. The fractions were collected and tested for conductivity or 100 μl tested for LT activity. All fraction tubes received 200 μg BS carrier in PBS.

ionic strength buffer, this phenomenon could cause problems in the primary resolution of the various human LT classes in a separation protocol. Indeed, if a similar situation occurs with LT from other animals species, it could account for differences between the reported sizes of these materials released by lymphoid cells from the same animals but studied in different laboratories (13, 14). The capacity of human LT molecules to form complexes and the importance of these complexes as lytic effectors is more thoroughly discussed in the third manuscript of this series.

The amount and class of LT detectable in a given supernatant or pool is influenced by a number of variables: (a) lability of certain classes; (b) culture conditions and the presence of absence of serum; and (c) the nature of the activating stimulus (2, 19, 27). A general observation has been that the higher the molecular weight, the more stable are the classes or subclasses of LT to heating, storage and fractionation. Complex and members of the α class are stable to freezing for long intervals, even when fractionated. However, β and γ classes in serum-containing media are quite labile and not stable to handling or storage. In our media, complex, α classes, and β_2 subclass, are stable to 56°C for several hours, while β_1 and γ-LT activities are rapidly inactivated. All LT molecules have proven stable in this medium when frozen for up to $1\frac{1}{2}$ years. However, upon fractionation, β_1 rapidly losses activity.

The human LT classes each appear to contain a number of subclasses. The large molecular weight complex class is heterogeneous. This is evident from its complex profiles after separation on DEAE and PAGE. The components of this high molecular weight LT have been carefully studied, and the properties are described in a separate manuscript (29). Alpha class LT activity contains at least six subclasses, separable by ion exchange chromatography and PAGE. These have been termed α_1, α_2, and α_3, on the basis of the order of their elution from DEAE. It is apparent that the α_2 subclass can be further resolved into multiple components by further chromatography on phosphocellulose columns. Those materials which are

not retained on PC are termed α_{2a} and those materials which stick are termed α_{2b}. The number of resolvable components is dependent upon the pH and slope of the eluting salt gradient. At pH 6.6, only two peaks, termed 2a and 2b were noted, whereas at pH 5.5, additional peaks were resolved. While the amounts vary, between 70% and 85% of LT associated with the α-LT class resides in the α_2 subclass. Alpha$_1$ and $_3$ subclasses represent about 20 and 10% of the remaining activity, respectively. The α subclassses appear to be clearly separable one from another by these procedures. In support of this concept, we find a single peak of radioactivity in the α_1 DEAE fraction with identical rf values of α_1 activity, if we fractionate supernatants of mitogen activated human lymphocytes cultured in ^{14}C amino acids. The β class contains two subclasses which, while similar in size, are quite different in charge and stability. Upon fractionation, β_1 is weakly charged, fr = 0.28 to 0.30, and unstable to both heating at 56°C and storage at − 10°C, whereas, β_2 is more highly charged, rf 0.46 to 0.50, and is stable to both heating and storage. The degree of chemical purity of these fractions is difficult to assess in the present studies, because the protein concentration in samples after the molecular sieving step(s) is almost nondetectable. Degrees of chemical purity of these fractions are presently being determined by isotopic methods.

Other investigators have examined the physical-chemical properties of lymphotoxins released by lectin activated human lymphocytes *in vitro*. The initial studies on human LT activities centered about stable members of the predominant 70,000 to 90,000 MW α class (17, 18, 25). The other classes were missed for a variety of technical reasons previously mentioned. Studies conducted by Walker *et al.* (19), Hiserodt *et al.* (27), and Lee and Lucas (21) on supernatants from PHA stimulated human tonsil and adenoid lymphocytes first suggested the true complexity of the human LT system. These authors demonstrated the existence of α and β class activity, and the latter separated the α class activity into three subclasses, two identified on phosphocellulose, α_1 and α_2, and they further resolved α_1 into two components on DEAE, termed 1a and 1b. The present studies indicate the human α-LT class is more heterogeneous than previously described. We feel the difference between our findings and those of others is because we (a) employed various modifications of the separation procedures, *i.e.*, variation of pH and more shallow elution gradients, which facilitated resolution of materials not previously noted; (b) we worked rapidly with these materials; and (c) we employed a specialized culture medium which helped to stabilize the smaller MW LT components. These studies further describe the complexity of the human LT system with identification of the complex and γ classes, and subfractionation of the β class into subclasses.

The human LT system, while heterogeneous, exhibits consistency in the number of classes and subclasses of materials detected. An important question which will be addressed directly in the subsequent study in this series is what are the relationships of these materials one to another. One source of molecular heterogeneity of soluble phase LT molecules may be the particular lymphoid cell type, i.e., T, B, or null, involved in release of the material. We have found that human T and B cells, while both capable of releasing LT upon stimulation with mitogens *in vitro*, may release different classes (30). This concept is supported by the finding that continuous human lymphoid cell lines of B cell origin appears to only release members of the α class of LT (9, 10, 31). A second source of heterogeneity of soluble phase LT activity is due to the activating agent itself, for the proportions of the different

classes vary dramatically with the type of activating agent employed (unpublished observations).

The human LT system appears much more complex when we compare it to the molecular features of LT described in studies of these materials from experimental animals. These relationships have been discussed elsewhere (21, 32), however, only material(s) of either α or β MW class have been described. This situation is now clarifying, as will be reported in another manuscript, that multiple classes somewhat similar to those described for the human are also found in supernatant from lymphocytes from other animal species when the cells are activated under the appropriate conditions (33). This study demonstrates that materials with LT activity in supernatants from PHA activated human lymphocytes *in vitro* are very heterogeneous. While complex, the situation is beginning to clarify, for in subsequent manuscripts it will be shown these materials form an interrelated system of molecules.

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