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Isolation and Identification of an α_2 Subclass Lymphotoxin (LT) Subunit from the High-Molecular-Weight (Complex) Human LT Class

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The high-molecular-weight (MW) complex class (MW = ≥200,000) of lymphotoxin (LT) may be involved in the process of lymphocyte-mediated cytolysis. This LT class was produced by concanavalin A-stimulated human adenoid and tonsil lymphocytes *in vitro* and purified ~1000 fold by a scheme employing lectin affinity chromatography on concanavalin A-Sepharose, negative hydrophobic affinity chromatography on phenyl-Sepharose, and molecular sieving on Ultrogel AcA 44. A cell-lytic subunit, termed C-alpha toxin, was dissociated from this partially purified complex LT (Cx) preparation. The identification of C-alpha toxin as an α_2 LT form was established by its comigration with the α_2 LT form during native polyacrylamide gel electrophoresis (PAGE), molecular sieving, and isoelectricfocusing. In addition, C-alpha toxin was neutralized by antiserum raised against purified α_2 LT, and the latter was neutralized by antiserum raised against C-alpha toxin. Furthermore, preliminary evidence indicates that both α_2 LT and C-alpha toxin are composed of peptides with apparent MW of ~69,000. Additional data indicate the Cx LT form is composed of α_2 LT and other subunits. © 1984 Academic Press, Inc.

INTRODUCTION

Lymphotoxins (LT) are a family of cytolytic and cytostatic glycoproteins released *in vitro* by lectin and antigen stimulated lymphocytes, as well as by certain lymphoid cell lines (1, 2). Lymphotoxins from both man and experimental animals are heterogeneous and can be separated by molecular weight (MW) into different classes (3). The human alpha (α) (MW 70–90,000) and beta (β) (MW 30–50,000) classes predominate in most lymphocyte supernatants and have been further separated by ion exchange chromatography into charge subclasses termed α_1, α_2, α_3, β_1, and β_2 (4, 5). Immunological studies have shown that several LT classes are related (6).

A large MW LT form, termed complex (Cx) (MW ≥200,000) has been identified in supernatants from lectin stimulated human T lymphocytes by molecular sieving

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and velocity sedimentation in sucrose gradients (7). When the Cx forms are subjected to molecular sieving in high ionic strength buffers, they dissociate into toxins with MW similar to the α heavy (α_H) (140–160,000 MW) and α LT classes, suggesting that the larger subclasses are a noncovalent assemblage of smaller LT subunits (7). To date attempts to reassemble Cx LT forms from the lower MW subunits have not been successful.

Functional and serological properties distinguish Cx and α_H LT from the lower MW LT classes. Two studies have indicated that the Cx and α_H classes of LT obtained from human and murine lymphoid cells are more effective cytotoxins *in vitro* than the lower MW α and β class forms (8, 9). Human high MW LT classes are recognized by antisera directed against human $F(ab')_2$ IgG fragments and these IgG-like components of LT are produced by T lymphocytes (10, 11). Finally, Ware and Granger have reported that antibodies directed against partially purified preparations of human Cx LT inhibit cytolysis mediated by human cytotoxic T cells *in vitro,* while antisera directed against human α and β LT have little effect (12, 13). These properties of Cx LT have led to the proposal that it is involved in lymphocyte mediated cytolysis. Although the mechanism of this process has attracted a good deal of interest, Cx LT itself has received little attention because it is both unstable and present at very low levels in lymphocyte supernatants.

This paper reports the identification of α_2 LT as a noncovalently associated subunit of human Cx LT and the partial purification of this high MW LT class. We were interested in whether α LT was a subunit of Cx LT since α LT is produced by some B cell lines which do not produce Cx LT (2). Alternatively the smaller cytotoxins derived from Cx LT may have been degradation products of a larger cytotoxic component or a toxic subunit similar only in size to α LT. The finding that α_2 LT is a subunit of Cx LT raises the question of why this or LT is assembled into the high MW Cx LT by T lymphocytes is discussed.

MATERIALS AND METHODS

Cell lines and culture media. Murine α-L-929 cells were passed biweekly in RPMI 1640 supplemented with 3% fetal calf serum as previously described (14).

Production of LT containing supernatants. Lymphocyte supernatants containing Cx LT were produced by the stimulation of human adenoid and tonsil lymphocytes with phytohemagglutinin-P (PHA) in serum-free media as previously described (10). The serine esterase inhibitor α-toluenesulfonyl floride (Eastman, Rochester, N.Y.) was added to the lymphocyte supernatants to give a final concentration of 1 m*M* and the supernatants were stored at −20°C. All purification procedures were carried out at 4°C. The α LT used in these studies was prepared from human lymphocytes and lymphoid cell lines, as previously described (15, 16).

Lymphotoxin assay. These techniques have been described in detail previously (14). One unit of LT activity is defined as the amount of activity necessary to lyse 50% of nondividing L-929 target cells (10^5 cells/tube in 1.0 ml).

Antisera. C-Alpha toxin was prepared by subjecting Cx LT (which had been purified first by Con A and phenyl-Sepharose chromatography and then by molecular sieving on AcA 44, as described below) to isoelectricfocusing on a pH 3.5–10 gradient. Immediately after the focusing column was drained, 3 ml from the pH 6.4–6.6 region (which contained the peak C-alpha toxin activity) was homogenized

with an equal amount of Freund's complete adjuvant (GIBCO) and injected intradermally at multiple sites on the back of a 6- to 7-week-old female New Zealand white rabbits as described by Vitakaitus *et al.* (17).

The production of rabbit antisera to the purified α_2 subclass of human LT and to the F(ab')$_2$ region of human immunoglobulin has been previously described (6, 7).

Antibody neutralization of LT. The percentage neutralization of cytotoxicity was determined by mixing 1–2 units of LT with different amounts of either antiserum or normal serum; after 20 min this mixture was assayed on L cells and the degree of neutralization calculated as previously described (6).

Radioiodination. Proteins were iodinated with ^{125}I using 5 to 10 μg of Iodogen (Pierce, Rockford, Ill.) as previously described (15, 16).

The Na ^{125}I (ICN, Irvine, Calif.) was diluted to 1 mCi per 50 μl with phosphate-buffered saline, pH 7.4. Protein solutions (100–500 μl) were added to freshly prepared tubes containing the Iodogen then 25 to 50 μl of Na ^{125}I was added. After various labeling intervals, the free iodine was removed by the technique of Tuszyski *et al.* (20).

Protein determination. Protein concentrations in initial supernatants were determined by the fluorescamine assay as previously described (21) and by the method of Bradford (22) using bovine serum albumin as a protein standard. Reagents for the Bradford assay were purchased from BioRad (Richmond, Calif.); fluorescamine was purchased from Sigma (St. Louis, Mo.). All lymphocyte supernatants and fractions from various biochemical procedures were trace labeled with ^{125}I to facilitate the monitoring of protein during sequential purification procedures because the protein was only detectable by fluorescamine in the initial phases of these separation procedures. In most cases, fractions containing LT activity were relabeled with ^{125}I between each separation procedure for the actual amount of LT in these early step(s) was so low, compared to non-LT proteins, that little or no label was introduced into the LT molecule(s) itself. A Beckman (Fullerton, Calif.) Biogamma Counter was used to quantitate ^{125}I.

Cytotoxin containing fractions were relabled between the various stages of the purification scheme in order to maintain an appropriate level of radioactivity.

Lectin affinity chromatography. Columns containing 10 ml of packed concanavalin A (Con A)-Sepharose (Pharmacia, Piscataway, N.J.) were first washed with 10 bed volumes of 10 m*M* phosphate (pH 7.2), loaded with lymphocyte supernatant, washed with 10 bed volumes of 10 m*M* phosphate (pH 7.2), and finally eluted with 10 bed volumes of 100 m*M* methyl-α-D-glucopyranoside (MGP) (Sigma, St. Louis, Mo.) in 10 m*M* phosphate (pH 7.2). The flow rate during these procedures was ~0.3 bed volumes per minute. The columns were then washed with 5 column volumes of 2 *M* MGP in 80% ethylene glycol (Mallinckrodt, Paris, Ky.) to remove residual protein.

Hydrophobic affinity chromatography. Pentyl to octyl-agarose columns were prepared by reacting the corresponding amines with carbonyl-diimidazole activated agarose (Reacti-Gel, Pierce, Rockford, Ill.) and equilibrating them in 10 m*M* phosphate (pH 7.2). An ^{125}I labeled, Con A-Sepharose binding, fraction of lymphocyte supernatant was loaded on each of these columns as well as on a phenyl-Sepharose column (Pharmacia, Piscataway, N.J.) and a benzyl-Agarose column (Pierce, Rockford, Ill.). These columns were washed with buffer containing 10 m*M* phosphate

(pH 7.2) and 100 m*M* MGP; the amount of radioiodinated protein and lytic activity that appeared in the breakthrough fractions was determined.

Ultrafiltration of LT containing preparations. After phenyl-Sepharose chromatography the breakthrough fractions were concentrated 50-fold prior to molecular sieving with a 50–90% yield; however, after isoelectricfocusing, >90% of the lytic activity and radioiodinated protein was lost during concentration by ultrafiltration on PM 10 membranes. By using YM10 membranes and either bovine serum albumin at 1 mg/ml or polyethylene glycol-4000 (Baker Chemical Co., Phillipsburg, N.Y.) at 0.1% this loss was reduced to 40%.

Molecular sieving. Columns containing degassed Ultrogel AcA 44 (LKB, Uppsula, Sweden) (65 × 2.5 and 35 × 1.4 cm) were poured and equilibrated with 20 m*M* phosphate, pH 7.2 and run as described previously (4). A maximum of 2 ml was loaded on the 65 cm column and 100 drop fractions were collected. A maximum of 600 μl was loaded onto the 35 cm column and 26 drop fractions were collected. A 42 × 1.4-cm P-60 column was poured and equilibrated in a similar fashion. A maximum of 500 μl was loaded on this column and 46 drop fractions were collected.

Electrophoresis. Reducing and nonreducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described by Laemmli (23). Native discontinuous PAGE was performed as described by Davis (24). After electrophoresis, the 7 cm native tube gels were immediately sliced into 1 mm sections; lytic activity was eluted from these gels by placing four adjacent slices in a siliconized Biogamma vial (Beckman, Fullerton, Calif.) with 600 μl of RPMI 1640 containing 1 mg/ml of bovine serum albumin. These tubes were then agitated gently at 4°C; after 8–12 hr, 200-μl samples of supernatant were removed from the tubes and assayed in duplicate on L cells.

Immunoprecipitation. Radioiodinated samples of protein (20–500 μl) were placed in 1.5 ml microfuge tubes with 10 to 20 μl of antiserum or control normal rabbit serum. This mixture was incubated at 4°C; after 30 min to 8 hr, protein A-Sepharose beads (Sigma, St. Louis, Mo.) (enough to bind 400 μg of human immunoglobin G) were added in a 100–160 μl volume. This mixture was then gently agitated at 4°C; after 30 min to 8 hr, the Sepharose beads were washed 3 times with 1 ml of a solution containing 150 m*M* NaCl, 0.05% NP-40, and 10 m*M* phosphate pH 7.2. The beads were then transferred to a new tube, washed once with 10 m*M* phosphate pH 7.2 and mixed with 70 μl of SDS–PAGE sample buffer.

Isoelectricfocusing. Isoelectricfocusing was performed in a 110 ml LKB preparative focusing column at 9°C using 1% ampholines and a 5–45% sucrose gradient; after reaching equilibrium fractions were collected and tested for bioactivity and radioactivity as described previously (25).

RESULTS

Preparation of Partially Purified Cx LT

Earlier studies had shown that Con A-Sepharose adsorbed lytic activity from preparations containing human α and α_H LT classes (21, 25, 26); therefore, the ability of this lectin to reversibly adsorb Cx LT from lymphocyte supernatants was examined. The typical profile in Fig. 1 shows that from 85 to 95% of all the LT bioactivity was adsorbed by the Con A-Sepharose. Virtually all of the adsorbed LT

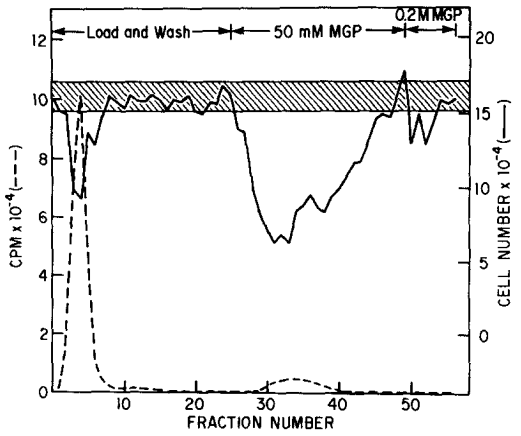

FIG. 1. Separation of lymphotoxin (LT) activity from lymphocyte supernatants by Con A-Sepharose affinity chromatography. A 30 ml volume of LT-containing supernatant was first trace labeled with ^{125}I and then loaded onto a 2 ml Con A-Sepharose column. The column was then washed with 100 ml of buffer, then eluted first with 120 ml of buffer containing 50 m*M* MGP, and finally with 50 ml of buffer containing 0.2 *M* MGP as described under Materials and Methods. Lytic activity was determined on L cells. The hatched area in this figure is the number of cells in control cultures.

activity could be eluted with 50–100 m*M* MGP. However, only 10 to 25% of the total radioiodinated protein adsorbed to the Con A-Sepharose columns, and only 10 to 15% of this adsorbed radioiodinated protein was eluted with 100 m*M* MGP resulting in a 40- to 100-fold purification. The use of a 0–200 m*M* MGP gradient rather than a single step elution with 100 m*M* MGP did not significantly reduce the amount of contaminating protein that desorbed with the LT.

A series of alkyl and aryl hydrophobic columns were screened in order to identify the matrix that could adsorb the greatest percentage of radioiodinated protein, while still allowing the LT activity to pass through the column unimpeded. The only two matricies tested that did not adsorb significant amounts of LT were phenyl-Sepharose and pentylagarose. Phenyl-Sepharose was chosen for further studies since we found it adsorbed more radioiodinated non-LT protein than the pentyl-agarose column. A profile shown in Fig. 2 illustrates the distribution of radiolabeled protein and lytic activity after Con A-Sepharose binding LT was passed through a phenyl-Sepharose column. In this experiment 80% of the radioiodinated non LT protein was bound by the phenyl-Sepharose column.

Lymphotoxin activity after separation on Con A- and phenyl-Sepharose columns was next subjected to gel filtration on AcA 44 columns. A typical column profile in Fig. 3A shows that about 14% of the radioiodinated protein loaded onto the column elutes with Cx LT in the void volume fractions of this column. In this experiment, the phenyl-Sepharose active fraction was relabeled with ^{125}I as described under Materials and Methods. The major peak of lytic activity in this column profile is α LT. Selected Cx LT containing preparations from the molecular sieving column were rechromatographed on AcA 44 to assure complete removal of lower MW material from these preparations. The profile resulting from the rechromatography of the complex LT containing fractions from Fig. 3A is shown in Fig. 3B. Samples from the Cx pool were radioiodinated and analyzed by native and SDS–PAGE;

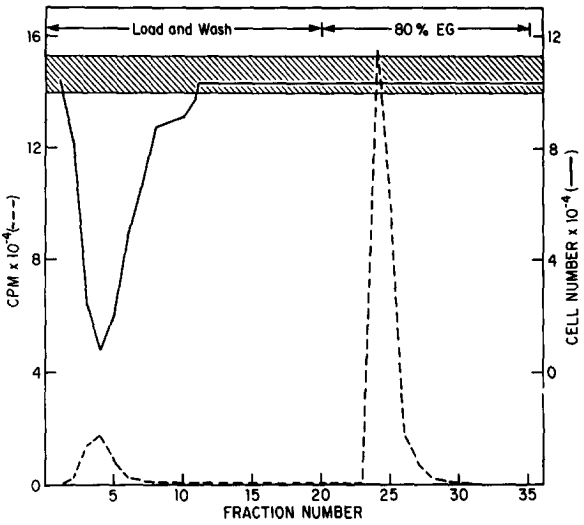

FIG. 2. Con A-Sepharose binding LT activity chromatographed on phenyl-Sepharose columns. A 2 ml column of phenyl-Sepharose was first loaded with 25 ml of Con A-Sepharose binding LT fraction that had been trace labeled with ^{125}I, then washed with 75 ml of buffer, and finally eluted with 25 ml of 80% ethylene glycol. Lytic activity was determined on L cells; the hatched region is the number of cells in control culture.

native 7% PAGE revealed that this preparation contained at least 10 separable components, and >20 peptides were seen in autoradiographs after reducing SDS–PAGE despite a 500- to 3000-fold purification.

Dissociation of α_2 LT from Cx LT

Fractions containing rechromatographed Cx LT from molecular sieving columns were subjected to isoelectricfocusing (IEF). In the experiment shown in Fig. 4, the LT and ~4% of the radioiodinated protein in the column focused in the region from pH 5.7 to 7.3. However, in other experiments the radioactive peak ranged from less than 0.5 to 6% of the total radioiodinated protein. The use of 5 to 8 or 4 to 8 pH gradients and increasing the focusing time to 44 hr resulted in no additional separation of the lytic activity from radioiodinated protein.

Preliminary experiments using AcA 44 columns had shown that after IEF the lytic activity resided in a molecule similar in size to α LT; this cytotoxin was initially termed C-alpha toxin. Further molecular sieving experiments were performed using a Bio-Rad P-60 column, which separates a narrower MW range of molecules, to more precisely compare α LT and C-alpha toxin. The results in Fig. 5 show that the majority of the radioiodinated protein was apparently lower in MW than C-alpha toxin, while a smaller peak of labeled protein eluted in the void volume fractions of this column. This column profile also demonstrates that C-alpha toxin and α LT co-chromatograph on Bio-Rad P-60.

The C-alpha toxin peak from IEF was also subjected to SDS and native PAGE. Under both reducing and nonreducing conditions multiple labeled peptides were observed in SDS gels. Since the LT bioactivity could not be recovered from gels containing SDS, preparations of C-alpha toxin were subjected to native PAGE to

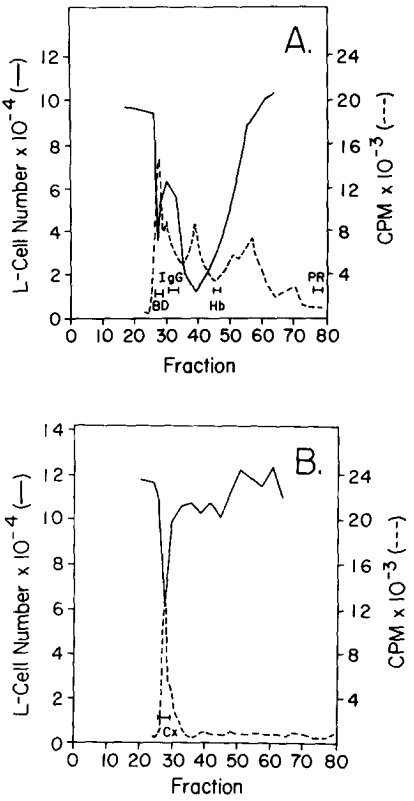

FIG. 3. The Con A-Sepharose binding LT activity from 1 liter of lymphocyte supernatant was passed through a phenyl-Sepharose column, concentrated to 2 ml, and chromatographed on a 65 cm AcA 44 column: (A) profile of LT activity and ^{125}I-labeled material from phenyl-Sepharose on AcA 44 columns; (B) rechromatography of the Cx peak on AcA 44. The complex LT that eluted in the void volume of Fig. 3A; indicated by the horizontal bar labeled BD, was concentrated to 2 ml and rechromatographed on the same AcA 44 column. The column was calibrated with molecular weight markers shown by the horizontal bars: blue dextran (BD) (2×10^6), immunoglobulin G (IgG) (15,000), hemoglobin (Hb) (64,000), and phenol red (PR) (393).

determine the relationship between the radioiodinated material and biologic activity. The results of one such experiment are shown in Fig. 6. In this 7% acrylamide gel the R_f of the lytic activity was 0.25 to 0.33; this is in agreement with the R_f of the α_2 subclass of human α LT in this PAGE system (4). However, there was little radioactivity in this region.

Antiserum raised against C-alpha toxin preparations partially neutralizes the lytic activity of both C-alpha toxin and α LT (Table 1). No additional neutralization was obtained by using higher levels of anti-C-alpha toxin. In addition anti-α_2 LT serum from rabbits immunized with highly purified material and anti-F(ab')$_2$ were tested for their ability to recognize C-alpha toxin. As shown in Table 1, antiserum raised against α_2 LT recognized C-alpha toxin. However, antisera raised against human F(ab')$_2$ which neutralizes Cx LT failed to neutralize C-alpha toxin. These antisera display the same pattern of reactivity with α LT (7).

The rabbit anti-α_2 LT serum was then used to immunoprecipitate C-alpha toxin from the IEF column for analysis by SDS PAGE. In the experiment shown in Fig.

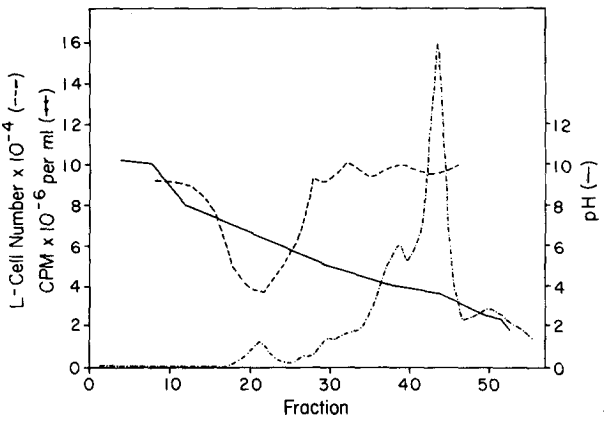

FIG. 4. Complex LT was prepared by passing the Con A-Sepharose binding material from 1 liter of lymphocyte supernatant through phenyl-Sepharose followed by molecular sieving on AcA 44. The complex pool from the AcA 44 was trace labeled with ^{125}I and subjected to isoelectricfocusing for 26 hr in 1% LKB ampholines. Lytic activity was determined on L cells as described under Methods and Materials.

7 only one peptide, with an apparent MW of 69,000, was specifically precipitated by the anti-α_2 nonspecifically adsorbed. Bands of equal intensity with apparent MW of 17,500 and 32,000 were observed in both the anti-α_2 and normal rabbit serum

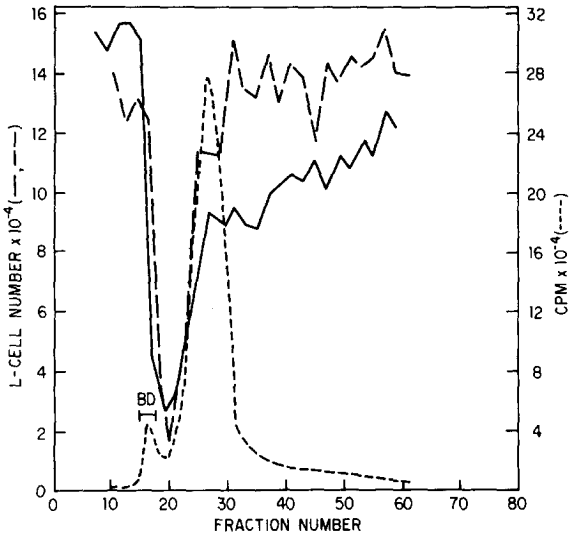

FIG. 5. C-alpha toxin and α_2 LT were compared by molecular sieving on P-60. C-alpha toxin fractions from a focusing column were pooled, concentrated, and chromatographed on a P-60 column. Alpha LT (MW ~80,000) from lectin stimulated lymphocytes was prepared by molecular sieving chromatography on an AcA 44 column as previously described (21). The alpha LT from these columns was rechromatographed to assure removal of all other MW LT classes. Then this sample was concentrated 10-fold and a 600 μl was chromatographed on the same P-60 column. The dashed line is C-alpha toxin and the solid line is the α LT sample. Lytic activity was determined on L cells; the void volume of the column is marked with blue dextran (BD).

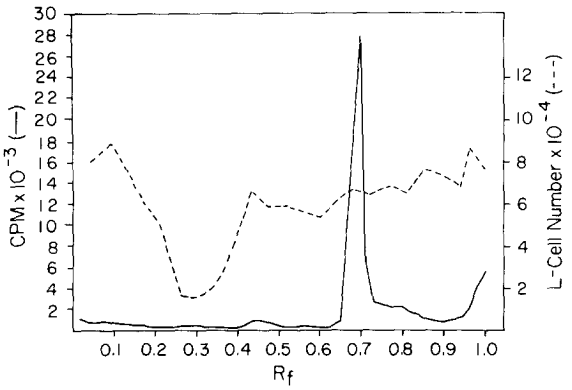

FIG. 6. C-alpha toxin from isoelectricfocusing was subjected to PAGE in 7% native gels. A 10 ml pool of the fractions containing C-alpha toxin from isoelectricfocusing was trace labeled with ^{125}I, concentrated 10-fold, and a 150 μl sample was subjected to native PAGE. After electrophoresis the gel was cut into 1 mm slices, each slice was eluted, and the eluate of each slice tested on L-929 cells for LT activity.

lanes of this experiment. The two smaller MW bands represent the major radiolabeled peptides in the IEF fractions. Because of the large amount of ^{125}I incorporated into these two peptides, the 69,000 MW peptide was practically undetectable when the IEF fraction was subjected to SDS–PAGE and autoradiography without immunoprecipitation.

DISCUSSION

The use of lectin affinity chromatography on Con A-Sepharose resulted in good separation of LT bioactivity from radiolabeled non-LT proteins. In view of the instability of the Cx form in even moderate ionic strength buffers (7, 10), we avoided ion exchange or positive hydrophobic affinity chromatography procedures. The second phenyl-Sepharose step following Con A-Sepharose chromatography resulted in a moderate separation of LT activity from radiolabeled non LT protein and gave a good yield of bioactivity. Molecular sieving on AcA 44 then separated

TABLE 1

Percentage Neutralization of C-Alpha Toxin and α LT by Various Antisera

	LT source[a]		
	Adenoid C-alpha toxin	Adenoid α LT	RPMI 1788 α LT
Anti-F(ab')$_2$	-5 ± 3	0 ± 1	3 ± 3
Anti-α_2 LT	101 ± 5	100 ± 2	106 ± 5
Anti-C-alpha toxin	53 ± 6	45 ± 3	49 ± 3

[a] Between 1 and 2 units of lytic activity and 25 μl of antiserum were added to each culture in these experiments.

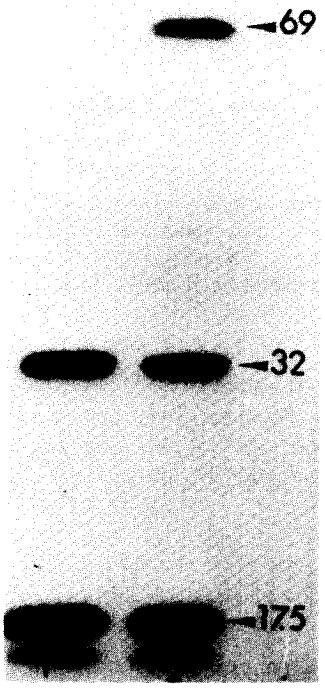

FIG. 7. Immunoprecipitation of C-alpha toxin after isoelectricfocusing. Immediately after the focusing column was drained, a 200-μl sample of the fraction containing peak C-alpha toxin lytic activity was iodinated, divided into two equal parts, and immunoprecipitated with normal rabbit serum (lane 1) or anti-α_2 LT (lane 2). The immunoprecipitate was then analyzed by SDS–PAGE in a 10% acrylamide separating gel under reducing conditions followed by autoradiography. The 32,000 and 17,500 MW bands represent the major iodinated species present in these fractions after isoelectricfocusing; they do not copurify with the cytolytic activity during PAGE or P-60 gel filtration. These molecules are not antigenically related to α_2 LT since, after washing as described under Materials and Methods, some of these molecules remain associated with the protein A-Sepharose beads whether anti-α_2 LT serum or normal serum are used.

Cx LT from the lower MW LT as well as from additional contaminating proteins. The approximate specific activity of the Cx LT produced by this scheme was ~1000-fold higher than the specific activity of the total LT activity in the initial lymphocyte supernatant. However, the degree of purification is only approximate since several assumptions were made in its calculation. For example, trace labeling with ^{125}I was used to determine protein concentration during the molecular sieving step, and Cx LT was assumed to have the same specific activity as the lower MW LT. Finally, this purification scheme assured that Cx LT would not be contaminated with lower MW LT forms.

Gel filtration chromatography demonstrated that C-alpha toxin was dissociated from Cx LT during isoelectricfocusing. This was possibly due to the ionic strength in the solutions in the focusing column or the presence of sucrose. It has been shown previously that Cx forms do not dissociate during velocity sedimentation in a 5 to 20% sucrose gradient which rules out the effect of sucrose (7). Alternatively, there may be dissassociation of the Cx LT and its subunits in the focusing column which would allow the free subunits to migrate to their individual isoelectric points, thereby, preventing the reformation of Cx LT.

Both biochemical and immunological characteristics of C-alpha toxin strongly suggest that this molecule(s) is a member of the α_2 subclass of human LT. The α_2 and C-alpha forms cochromatograph on P-60 molecular sieving columns, and they both have an R_f of $\sim$0.3 when subjected to electrophoresis in native 7% polyacrylamide gels. Additionally, they both have an isoelectric point of 6–7. While the R_f and isoelectric points distinguish C-alpha toxin and α_2 LT from α_1 and α_3 LT, it may be that the three subclasses of α LT differ only in the glycosilation of a core polypeptide. Finally, antiserum raised against α_2 LT neutralizes C-alpha toxin, while antiserum raised against the C-alpha toxin partially neutralizes both α LT and C-alpha toxin. The failure of anti-C-alpha toxin serum to completely neutralize the lytic activity of C-alpha toxin and α LT forms suggest that the rabbit did not respond against all of the subpopulations of C-alpha toxin molecules, or that this is a weak antiserum.

The data currently available on the polypeptide composition of α_2 and C-alpha toxin also indicate these molecules are quite similar. Anti-α_2 specifically precipitated single ^{125}I-labeled peptide(s) with apparent MW of 69,000 on SDS gels from preparations of C-alpha toxin. When α_2 LT from human adenoid lymphocytes was purified in our laboratory (21) and subjected to SDS–PAGE tube gels under reducing conditions, a peak of radioiodinated protein with apparent MW of 68,000 to 70,000 was observed (unpublished observation). Additional studies indicates that purified α_2 LT from a human lymphoblastoid cell line, IR 3.4, when labeled with ^{125}I appears as a single peptide with an apparent MW of 68–70,000 in reducing SDS gels (16).

If C-alpha toxin is not the degradation product of some larger component of Cx or a cytotoxic subunit similar only in MW to α LT, but is actually α_2 LT, what can be said about the precursor–product relationship of α_2 and Cx LT? Previous investigators have been unable to reform Cx LT by combining the lower MW LT classes (7). On the other hand, this study has shown that Cx LT can dissociate into the α_2 LT form; however, it does not seem likely that all α_2 LT detectable in a supernatant is derived from Cx LT precursors since Cx forms have never been identified in the supernatants of α_2 LT-producing human continuous cell lines of B lymphoid cell origin. Since antisera raised against both β_1 LT and F(ab')$_2$ fragments of human IgG react with Cx LT, but not with α LT (7), it is likely that Cx LT comprises other subunits in addition to α_2 LT. Together, these data suggest that Cx LT can be assembled either intracellularly or on the plasma membrane, but not in the supernatant.

Once assembled, Cx LT may be transferred directly from the membrane of a cytotoxic lymphocyte to the membrane of a target cell. Those molecules which are not transferred to the target cell membrane may then dissociate into less toxic low

MW LT, thus preventing nonspecific cytolysis. Several recently published studies contain electron micrographs showing that a large molecule(s) may be inserted into target cell membranes by cytotoxic T cells and natural killer cells (27, 28). However, the relationship of these molecules to Cx LT remains to be determined.

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