Human Lymphotoxin

PRODUCTION BY A LYMPHOBLASTOID CELL LINE, PURIFICATION, AND INITIAL CHARACTERIZATION

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Human lymphotoxin was purified to homogeneity from a serum-free tissue culture supernatant of a lymphoblastoid 1788 cell line. The purification scheme consisted of DEAE-cellulose chromatography, preparative isoelectric focusing, lentil lectin-Sepharose chromatography, and preparative polyacrylamide gel electrophoresis. The purified glycoprotein was homogeneous by the criteria of high pressure liquid chromatography and polyacrylamide gel electrophoresis run under both nondenaturing and denaturing conditions. The specific activity of the purified lymphotoxin is approximately 40 x 10^6 units/mg. The protein has an apparent molecular weight of approximately 20,000, an Rf of 0.33 on 7.5% polyacrylamide gels at pH 8.8 and an isoelectric point of 5.8. A tryptic digest of the purified native material produced two major fragments of approximately 15,000 and 5,000 Da. The amino acid compositions of the intact molecule and of the tryptic fragments are presented.

Lymphokines are hormone-like substances produced by stimulated lymphocytes and have been implicated in the regulation of the immune system (1). Lymphotoxin is a lymphokine which specifically inhibits tumor cell growth in vivo (2-7) and in vitro (8-10) and is usually less cytostatic against nontumorigenic cells from the same species. (11-15). Lymphotoxin has been shown to inhibit both the transformation of cells induced by chemical carcinogens or ultraviolet radiation (16, 17) and the ability of morphologically transformed cells to produce tumors in vivo. Most of these biological studies have been carried out with relatively crude lymphokinin preparations. A number of laboratories are involved in the purification of lymphotoxin (18-21), the source of which has been human tonsil and adenoid-derived lymphocytes, human peripheral blood lymphocytes, and lymphocyte cell lines. Progress has been limited primarily because of the minute quantity of active material produced by these sources. Therefore, there is very little information available on the biochemical properties of lymphotoxin. In this report, we describe the purification of lymphotoxin from a human lymphoblastoid cell line and some of the physical and chemical properties of the purified protein.

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EXPERIMENTAL PROCEDURES

**Determination of Biological Activity**—The activity of lymphotoxin during purification was monitored by a previously described modified cell-lytic assay (22). Briefly, mouse L-929 fibroblast cells (obtained from Dr. Gale A. Granger, University of California, Irvine, California) were grown on microtiter plates in the presence of 0.75 µg/ml of mitomycin C (Sigma). After 12-18 h, these cells were exposed to 0.125 ml of a serially diluted test sample of lymphotoxin. The test sample was removed after 48 h, the plates were washed, and cell lysis was detected by staining the plates with a 0.5% solution of Crystal Violet in methanol/water (1:4) (v/v). The end point on the microtiter plates was determined by a Microtiter autoreader (Dynatech) set for absorption at 450 nm and transmission at 570 nm. The cells exposed to culture medium alone were set at 0% lysis and those exposed to a 3 mM guanidine hydrochloride solution provided an end point for 100% lysis. One unit of lymphotoxin is defined as the amount required for 50% cell lysis of 12,000 cells plated in such well.

**Protein Determination**—Protein concentration was determined by the method of Bradford (23). During the final stages of purification, the protein concentration was estimated by absorbance at 280 and 206 nm and also by amino acid analysis.

**Tissue Culture**—The human lymphoblastoid cell line RPMI 1788 was obtained from ATCC (No. CCL 156) and the seed culture was grown in 2-liter roller bottles using 400 ml of RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) containing 10 mM HEPES and 5% fetal calf serum at a cell density of 1 x 10^6 cells/ml. After 5 days at 37°C, when the culture reached a cell density of 2 x 10^7/ml, the cells were harvested, washed twice with serum-free RPMI 1640 medium, and then transferred into the same medium containing 10 mM HEPES and 1% penicillin/streptomycin at a final cell density of 5 x 10^6 cells/ml. Cells were grown in suspension in 2-liter roller bottles and in 15-liter flasks (Belco). After 60 h, the cell supernatants were harvested by passing the media through a 3-µm Seakleen filter (Pall Trinity Micro Corp., Cortland, NY). The clear filtrate was assayed for lymphotoxin activity and used for subsequent purification and characterization.

**Concentration and Dialysis of Filtrates**—The cell filtrates were concentrated approximately 20-fold at 4°C on a Pellicon polysulfone membrane (Millipore) with a molecular weight exclusion limit of 10,000 and simultaneously dialyzed against 5 mM sodium phosphate buffer at pH 7.8. When the conductivity of the sample reached that of the dialysis buffer, the membrane was washed thoroughly with buffer and any protein which was recovered was pooled with the concentrate.

**DEAE-52-cellulose Chromatography**—The dialyzed protein concentrate was applied to a DEAE-cellulose (Whatman) column (2.5 x 50 cm) equilibrated in 5 mM sodium phosphate buffer at pH 7.8 with a flow rate of approximately 1 liter/h. After the sample was loaded at 4°C, the column was washed with equilibration buffer and eluted at room temperature with a linear gradient of 0-0.3 M sodium chloride in 5 mM phosphate buffer. The eluates were monitored for absorbance at 280 nm, lymphotoxin activity, and for conductivity using a CDM 2E Conductivity Meter (Radiometer).

1 The abbreviations used are: HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HLT, human lymphotoxin; HPLC, high pressure liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; α-MM, α-methylmannoside; PBL, peripheral blood lymphocytes; Tris, 2-amino-2-hydroxyethylpiperazine-1,3-diol.
Preparative Isoelectric Focusing—The active fraction from the DEAE column was pooled, concentrated by ultrafiltration on a PM-10 membrane (Amicon), and dialyzed against 2.5 mM Tris and 20 mM glycine at pH 8.2. The concentrate was mixed with a dextran gel containing carrier ampholytes (pH 5–8) (LKB) and poured onto an isoelectric focusing flat-bed apparatus (10 × 24 cm) (LKB Multiphor). Electrofocusing was run along its length with a constant power of 8 watts for 15–20 h at 8 °C. The focused protein fractions were collected by sectioning the gel bed and eluting each section in small plastic columns with 50 mM ammonium bicarbonate buffer. The eluates were monitored for pH, absorbance at 280 nm, and for lymphotoxin activity.

Lentil Lectin Chromatography—The pooled activity from the previous step was directly applied to a lentil lectin-Sepharose 4B (2 mg of lectin/ml of gel) (Pharmacia) column (1 × 5 cm) equilibrated in 5 mM phosphate buffer at pH 7.8 and 25 °C. The column was washed with equilibration buffer and eluted with the same buffer containing 100 mM α-methylmannoside (Calbiochem).

Native Polyacrylamide Gel Electrophoresis—Fractions containing lymphotoxin activity from the lentil lectin column were analyzed by discontinuous native polyacrylamide gel electrophoresis which primarily consisted of the Laemmli system (24) poured and run in the absence of sodium dodecyl sulfate. Both preparative (1.5–4-mm thick) and analytical (0.75-mm thick) slab gels were used with 7.5% acrylamide in the resolving gel and 4% in the stacking gel. The running buffer was 25 mM Tris and 0.19 mM glycine at pH 8.4. The sample was applied in 15.6 mM Tris-HCl at pH 6.8. The analytical gel was run at a constant current of 22 mA and the preparative gel was run at 100 mA and constant temperature (7 °C). The protein on the gel was visualized by silver staining (25). Wherever necessary, the gels were sliced before staining and lymphotoxin activity was eluted by incubating the slices overnight at 4 °C in 50 mM ammonium bicarbonate buffer.

Reverse Phase High Pressure Liquid Chromatography—The active fraction from the preparative native polyacrylamide gel electrophoresis was concentrated by ultrafiltration with a PM-10 membrane and applied to a Spectra Physics SP 8000 high pressure liquid chromatograph equipped with a Synchropak RPP column (25 cm × 4.1 mm, Synchropak, Inc., Linden, NJ). The elution conditions consisted of a linear gradient of 1–70% acetonitrile in 0.1% trifluoroacetic acid at 25 °C and a flow rate of 1 ml/min. The effluent was monitored at an absorbance of 210 nm, absorbing peaks were collected, and the protein was lyophilized and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (24).

Molecular Weight Determination—The molecular weight of lymphotoxin was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by gel permeation chromatography. Fifteen percent polyacrylamide gels were run in the presence of SDS according to the procedure of Laemmli (24) and proteins were visualized by silver staining (25). High pressure gel permeation chromatography was carried out at room temperature using a TSK 3000 SW (Altek, Inc.) column (7.5 × 60 mm) run in 0.2 M sodium phosphate buffer, pH 7.8, at a flow rate of 0.5 ml/min. The column was calibrated with bovine serum albumin (M, 67,000), ovalbumin (M, 45,000), chymotrypsinogen (M, 25,000), and cytochrome c (M, 12,500). The molecular weights of lymphotoxin and of tryptic-derived peptides were also calculated from amino acid composition data using the method of Black and Hogness (26).

Tryptic Digestion and Peptide Isolation—The purified lymphotoxin preparation was digested with tosophenylalanyl chloromethyl ketone-trypsin ( Worthington) using 1 part trypsin to 20 parts lymphotoxin by weight in 50 mM ammonium bicarbonate buffer at pH 8 for 24 h at room temperature. At the end of hydrolysis, the tryptic peptides were separated on a Lichrosorb RP-18 column (25 cm × 4.6 mm, EM reagents, Cincinnati, OH) at 25 °C, using a Spectra Physics SP-8000 chromatograph. The peaks were detected at 210 and at 280 nm after elution with a linear gradient of 1–70% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. For amino acid composition and for SDS-polyacrylamide gel electrophoresis, each peak was collected and the material was lyophilized.

Amino Acid Analysis—The amino acid composition of intact lymphotoxin and the tryptic-derived peptides was obtained on a Beckman 6300 amino acid analyzer equipped with ninhydrin detection and a Hewlett-Packard 3590A integrator. The samples were dissolved in 0.2 N cold constant boiling HCl in acid-washed hydrolysis tubes, sealed under reduced pressure, and hydrolyzed at 110 °C for 24 h. Cysteine was determined by reduction with dithiothreitol in the presence of 8 M urea and alkylation with iodoacetic acid (27).

RESULTS

Purification of Human Lymphotoxin from 1788 Lymphoblastoid Cell Culture Medium—The 1788 human lymphoblastoid cell line was cultured in a serum-free medium for 65 h and 50–100 liters of cell supernatant were harvested per week by filtration. After concentration and dialysis, the supernatants had a specific activity of approximately 3,000 units/mg of protein, and at this stage, fractions of lymphotoxin activity from several separate harvested supernatants were pooled and applied to DEAE-cellulose. The lymphotoxin activity quantitatively bound to the DEAE-cellulose column and was eluted with a 0–0.3 M linear sodium chloride gradient (Fig. 1). A single sharp peak of activity was observed which eluted at approximately 0.1 M NaCl and represented a 47-fold increase in specific activity with an 80% recovery. This active fraction was concentrated, dialyzed, and further purified by flat-bed isoelectric focusing in the pH range 5–8. The activity, protein, and pH profile obtained are shown in Fig. 2. The lymphotoxin activity was observed in the pH range of 5.5 to 6.5. The preparative isoelectric focusing provided approximately a 2-fold purification and 60% recovery of the activity.

The active fraction from the isoelectric focusing step was applied to a lentil lectin-Sepharose column. As shown in Fig. 3, greater than 95% of the total protein with no lymphotoxin activity filtered through the column, and washing with column buffer (10 mM sodium phosphate buffer at pH 7.8) did not elute the activity. When the column was exposed to 100 mM α-methylmannoside in the presence of the column buffer, the lymphotoxin activity was eluted in a region having very little absorbance at 280 nm. A small percentage of residual activity was eluted with 0.5 M α-methylmannoside. A 23-fold purification with quantitative recovery of lymphotoxin activity was achieved.

![Fig. 1. DEAE-cellulose column chromatography of lymphotoxin showing bioactivity (O—O), protein (P—P), and conductivity (O—O) profiles. The column (2.5 × 50 cm) was equilibrated in 5 mM phosphate buffer, pH 7.8, and eluted with 500 ml of a linear (0–0.3 M) sodium chloride gradient. The flow rate of the column was adjusted to approximately 1 liter/h for sample loading and 200 ml/h for elution of the lymphotoxin. The sample (4.3 × 10⁶ units in 13 liters) applied was concentrated and dialyzed cell supernatant from 294 liters of 1788 cell line conditioned culture medium. Fractions 21 through 32 were pooled and dialyzed for preparative isoelectric focusing.](http://www.jbc.org/Downloaded from)
Fractions 71 through 80 were pooled. Pposable plastic columns with 50 mM NH4HCO3, pH 8.0. The sample (2.2 X 106 units in 60 ml) was derived from DEAE step and contained 3.5 X 106 units in 100 ml. The lymphotoxin activity after isoelectric focusing was eluted by sectioning the gel into small disposable plastic columns with 60 mM NH4HCO3, pH 8.0. Fractions 8 through 15 were pooled. The peak eluted at pH 5.8 and had greater than 90% of the applied lymphotoxin activity was recovered in the molecular weight region of 20,000 and no activity was detected in the 60,000 region. However some activity was found at the dye front from cytotoxic components in the sample buffer. These results indicate that on SDS-PAGE purified lymphotoxin has a molecular weight of approximately 20,000 but around 60,000 on TSK-HPLC. The M, 60,000 form may represent an oligomer of lymphotoxin which can be dissociated under reducing conditions by SDS to monomer.

The purified, native lymphotoxin was also analyzed by analytical polyacrylamide gel electrophoresis. After silver staining, a very diffuse band was observed with an Rf value of 0.33. In a separate experiment, gels were sliced and eluted; the lymphotoxin activity was associated with a gel slice having an Rf value of 0.33. The eluates from these gel slices were subjected to SDS-PAGE, and those fractions which had lymphotoxin activity were subjected to SDS-PAGE under reducing conditions, a single protein band at a molecular weight of 20,000 was observed (Fig. 5). Similar results were obtained when the M, 60,000 bioactive TSK-HPLC pool was run on SDS-PAGE. In order to determine if the M, 20,000 band is associated with lymphotoxin activity, a control study was carried out first to determine the effect of SDS and β-mercaptoethanol on the lymphotoxin activity. It was found that 0.1% SDS treatment reduces lymphotoxin activity to about 10% of its original value and that the remaining activity is unaffected by 0.1 M β-mercaptoethanol. Thus, purified lymphotoxin was pre-treated with 0.1% SDS and 0.1 M β-mercaptoethanol, assayed, and then loaded onto SDS-polyacrylamide gels. After electrophoresis, the gels were sliced, eluted in 50 mM NH4HCO3 overnight, and assayed for lymphotoxin activity. A control lane with the sample buffer alone was run, sliced and assayed. The results are shown in Fig. 5. Greater than 90% of the applied lymphotoxin activity was recovered in the molecular weight region of 20,000 and no activity was detected in the 60,000 region. However some activity was found at the dye front from cytotoxic components in the sample buffer. These results indicate that on SDS-PAGE purified lymphotoxin has a molecular weight of approximately 20,000 but around 60,000 on TSK-HPLC. The M, 60,000 form may represent an oligomer of lymphotoxin which can be dissociated under reducing conditions by SDS to monomer.

Reverse phase HPLC (Fig. 6A) of purified lymphotoxin showed a single major protein peak which eluted with 50% acetonitrile. This peak was run on an SDS-polyacrylamide gel under reducing conditions and was found to have an approximate M, of 20,000. The HPLC solvents inactivated the lymphotoxin.

The trypptic digestion products of purified lymphotoxin were analyzed by SDS-PAGE (Fig. 7). The results indicate that digestion conditions with a trypsin to protein ratio of 1:20 by weight at 25 °C for 20-24 h were adequate for quantitative conversion of the M, 20,000 species to M, 15,000 and 5,000 bands. Furthermore, when the trypptic digest was applied to a reverse phase HPLC column, two distinct peaks, T-1 and T-2, eluted at acetonitrile concentrations of 42.3 and 48.4%, respectively (Fig. 6B). No protein was detected in the flow-through of the column. The M, of these 2 peaks as determined by SDS-PAGE (Fig. 7) were approximately 15,000 for T-1 and 5,000 for T-2. The lower absorbance at 210 nm of the T-1 fragment as compared to T-2 suggested low recovery of the T-1 peptide from the HPLC column and this was confirmed by quantitative amino acid analysis of the sample load and the resolved T-1 and T-2 peptides. The results of these
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TABLE I

| Purification step                          | Final volumes | Total protein | Cytolytic activity | Relative specific activity | Purification | Recovery |
|-------------------------------------------|---------------|---------------|--------------------|----------------------------|--------------|----------|
| Starting material                         | 294,000       | 4,560         | \(8.70 \times 10^6\) | 1,908                      | 1.6          | 49.0     |
| Concentration and dialysis                | 13,000        | 1,400         | \(4.30 \times 10^6\) | 3,071                      |              |          |
| DEAE-cellulose chromatography             | 208           | 24.50         | \(3.50 \times 10^6\) | 0.14 \times 10^6           | 75           | 39.7     |
| Preparative isoelectric focusing         | 100           | 8.60          | \(2.20 \times 10^6\) | 0.26 \times 10^6           | 135          | 24.6     |
| Lentil lectin chromatography              | 60            | 0.39          | \(2.30 \times 10^6\) | 5.9 \times 10^6            | 3113         | 25.6     |
| Preparative polyacrylamide gel            | 35            | 0.025         | \(0.97 \times 10^6\) | 39.0 \times 10^6           | 20,547       | 10.7     |
| electrophoresis                           |               |               |                    |                            |              |          |

Fig. 4. A profile of protein and bioactivity from high pressure liquid chromatography of purified lymphotoxin. Approximately 3 μg of native lymphotoxin in 0.5 ml of 50 mM ammonium bicarbonate was injected onto a TSK-3000 column (7.5 x 60 mm). The column was run in 0.2 M sodium phosphate buffer at pH 6.8 at a flow rate of 0.5 ml/min. Various fractions were pooled as indicated and bioassayed.

analyses are shown in Table II. The amino acid compositions of native lymphotoxin and of tryptic fragments T1 and T2 do not exclude the presence of other tryptic fragments. Overall, it appears that the intact lymphotoxin molecule consists of more acidic residues than basic amino acids, consistent with the isoelectric point of 5.8. Furthermore, the compositions of individual tryptic fragments indicate that T-1 is rich in lysine, arginine, and aspartic acid residues but low in methionine as compared to T-2. No cysteine was detected in native or reduced and carboxymethylated lymphotoxin. Based upon amino acid composition, the minimum molecular weights of native lymphotoxin and the trypsin-derived peptides T1 and T2 were 18,000, 12,500, and 6,300 respectively. The molecular weights of lymphotoxin and of tryptic fragment T2 obtained by this method were slightly lower than those obtained by SDS-PAGE. This may be due to the presence of carbohydrate residues in lymphotoxin.

DISCUSSION

The human lymphoblastoid cell line 1788, when grown in a serum-free medium, is known to secrete constitutively several lymphokines including lymphotoxin, macrophage and leukocyte migration inhibition factors, skin reactive factor, and macrophage activation factor (28). Lymphotoxin from the 1788 cell line has been purified to homogeneity by sequentially
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FIG. 6. Reverse phase high pressure liquid chromatography of purified lymphotoxin (top) and its tryptic digest (bottom). Approximately 2 μg of highly purified native lymphotoxin in 50 mM ammonium bicarbonate at a concentration of 0.1 μg μl⁻¹ was injected onto a Synchropak RPP C-18 column. The tryptic digest was prepared as described under “Experimental Procedures.” The elution solvents were 0.1% trifluoroacetic acid in water (solvent 1) and 0.1% trifluoroacetic acid in acetonitrile (solvent 2). Elution was with a linear gradient from 100% solvent 1 to 70% solvent 2 at 25 °C and a flow rate of 1 ml/min. The effluent was monitored at 210 nm. The major peaks were analyzed by SDS-PAGE.

using DEAE-cellulose chromatography, preparative isoelectric focusing, lentil lectin-Sepharose chromatography, and preparative polyacrylamide gel electrophoresis. Starting from approximately 300 liters of conditioned medium, the protein was purified 20,000-fold with a final recovery of 10.7%. The final preparation had a specific activity of 40 X 10⁶ units/mg of protein and was considered homogeneous by polyacrylamide gel electrophoresis under native and denaturing conditions, by reverse phase HPLC and by gel permeation HPLC.

Granger et al. (29) have observed lymphotoxin activity from different sources exhibiting molecular weights from 15,000 to greater than 200,000. Whether these various sizes of lymphotoxin are aggregates or breakdown products derived from a single molecular species has not been determined. The purified lymphotoxin isolated in the present study from a human lymphoblastoid cell line has a molecular weight of 60,000 by gel permeation chromatography. This molecular size may correspond to the α₁-form of lymphotoxin reported previously (29). Under denaturing conditions, the 60,000-Da molecule migrates as a 20,000-Da species. These results imply that native lymphotoxin has a molecular weight of approximately 60,000 and is dissociated to a 20,000 species by SDS under reducing conditions. Since we have directly observed a biologically active M₁, 20,000 and 60,000 species, we believe the M₁, 20,000 form to be a monomer. The observation of high and

FIG. 7. Sodium dodecyl sulfate-polyacrylamide (15%) gel electrophoresis of purified lymphotoxin and its tryptic fragments. The samples are from left to right: Molecular weight protein standards, purified lymphotoxin, tryptic digest of lymphotoxin, tryptic fragment T₁, tryptic fragment T₂, and trypsin. Before loading, samples were treated with 0.1% SDS, 0.1 M β-mercaptoethanol, and then heated at 90 °C for 1 min. Fragments were separated as described under “Experimental Procedures.”

| Amino acid residues | Intact molecule | T-1   | T-2   |
|---------------------|----------------|------|------|
| Aspartic acid       | 12.0           | 12.0 | 1.8  |
| Threonine           | 9.8            | 5.1  | 3.6  |
| Serine              | 21.3           | 15.7 | 7.4  |
| Glutamic acid       | 16.5           | 7.0  | 7.2  |
| Proline             | 11.8           | 7.0  | 5.2  |
| Glycine             | 14.3           | 9.0  | 4.4  |
| Alanine             | 12.6           | 10.3 | 4.4  |
| Cysteine*           | 7.0            | 4.0  | 3.1  |
| Valine              | 2.8            | 0.4  | 1.5  |
| Methionine          | 5.0            | 2.6  | 0.9  |
| Isoleucine          | 22.0           | 17.3 | 8.2  |
| Leucine             | 6.2            | 4.0  | 2.5  |
| Tyrosine            | 9.1            | 5.6  | 4.1  |
| Phenylalanine       | 6.8            | 3.9  | 2.7  |
| Histidine           | 7.7            | 6.8  | 1.0  |
| Lysine              | 3.3            | 3.7  |      |
| Arginine            | 18,330         | 12,500| 6,300|
| Molecular weight*   |                |      |      |

*None detected.

The minimum molecular weight was calculated from the amino acid composition (26), and the number of amino acid residues was calculated using this molecular weight.

TABLE II

Amino acid analysis of intact and tryptic fragments of purified human lymphotoxin

Protein samples were hydrolyzed in 0.2 ml of 6 N HCl for 24 and 48 h at 110 °C, lyophilized, and dissolved in 0.1 ml of 0.2 M sodium citrate (pH 2.2). The numbers presented represent the averages of quadruplicate determinations. ND, not determined.
low molecular weight forms of this molecule may have relevance to the heterogeneity in molecular weights of lymphotoxin observed by others.

There are other chemical similarities observed between the purified lymphotoxin reported here and the partially purified preparations of others. This includes the probable glycoprotein nature of lymphotoxin as suggested by lectin binding, acidic isoelectric point, and relative mobility on native polyacrylamide gels (29). The stability of the 1788 cell line-derived lymphotoxin towards pH and temperature is also similar to that previously reported for other crude human lymphotoxin preparations. Preliminary evidence has also indicated that rabbit antibodies made against 1788 lymphotoxin will neutralize the activity of peripheral blood lymphocyte-derived lymphotoxin induced by phorbol 12-myristate 13-acetate.2,3 Thus, all these observations allude to the similarity of the lymphotoxin molecule derived from different sources.

The purified native lymphotoxin, when exposed to trypsin, was cleaved into two major peptides, T1 and T2, indicating a single accessible trypsin-sensitive site in native lymphotoxin. The amino acid composition data, however, indicate that both the intact molecule and its tryptic fragments contain more than one trypsin-sensitive site. The presence of two major tryptic fragments, however, does not exclude the possibility of minor fragments which cannot be detected by SDS-PAGE or HPLC. This is also evident from the amino acid composition data. The molecular weights of tryptic fragments T1 and T2, determined by SDS-PAGE were 15,000 and 5,000, respectively; which agrees approximately with the values 12,500 and 6,500 obtained by amino acid analysis. Using a crude preparation, it has been reported that human lymphotoxin is resistant to trypsin (30). The tryptic digest of purified lymphotoxin reported here was also tested for biological activity. Under the conditions described, lymphotoxin bioactivity was not affected. The requirement of one or both of the tryptic fragments for biological activity has not yet been determined. At present, studies are also in progress to further characterize the molecule with respect to its primary structure, as well as its biological role.

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