Human Lymphotoxin: Purification and Some Properties
(phytohemagglutinin/human lymphocytes)

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Communicated by R. W. Gerard, September 25, 1972

ABSTRACT Lymphotoxin is secreted by human lymphocytes stimulated with phytohemagglutinin in vitro. Combinations of DEAE-cellulose and Sephadex chromatography, acrylamide gel electrophoresis, and isoelectric focusing were used to purify lymphotoxin 2000- to 4000-fold; 15-25% of the activity has been recovered. Lymphotoxin appears to be a weakly charged molecule(s) of molecular weight about 90,000-100,000 that migrates in Pevikon block electrophoresis as a β- or α-globulin. It is a discrete molecule(s), because it is completely separable from medium serum proteins and carrier and phytohemagglutinin proteins. Isoelectric-focusing studies indicate that there may be a limited heterogeneity among lymphotoxin molecules.

Lymphoid cells are central effectors in such important immunologic reactions as graft rejection (1), tumor immunity (2), and delayed hypersensitivity (3). All of these reactions appear to share at least two common steps: (a) a recognition step, which requires the interaction of the foreign tissue or graft cell with hypothetical lymphocyte-membrane receptor sites (4, 5) and (b) an effector step, which involves active cellular metabolism and biosynthesis (5). These events lead, by some unknown mechanism, to the destruction of the foreign cell.

Lymphoid cells from experimental animals and man can be activated in vitro specifically with antigens (6), or nonspecifically with mitogens, e.g. phytohemagglutinin (PHA) (7), to release various soluble molecules into the cell culture medium. These soluble molecules have been proposed to be effector substances associated with cellular immunity (6, 7). The treatments and agents that trigger the release of these factors are very broad and unrelated, yet they all induce similar changes in lymphocytes, namely, activation of RNA and protein synthesis and morphologic changes associated with transformation (8). One of these factors is a cell toxin termed lymphotoxin (LT). A few of the physical properties of a cell toxin released by normal human lymphocytes stimulated with PHA in vitro were reported (9). The toxin appeared to be a protein of molecular weight about 80,000 (9). This is a brief description of the methods for purification and some physical properties of LT released by human lymphocytes stimulated with PHA in vitro.

MATERIALS AND METHODS

Production of Human Lymphotoxin and Control Media. The details of these methods have been published (10). Cells were maintained in medium RPMI 1640 (from GIBCO), containing 0.1% bovine-serum albumin, at a density of 5 x 10⁶ cells per ml, in 32-oz prescription bottles, 200 ml per bottle, then 20 µg of PHA-P (Difco, Detroit, Mich., Lot no. 561822) per ml was added. After 5 days, the medium was collected, pooled, then cleared of cells and debris by centrifugation. Controls consisted of either lymphocytes cultured under identical conditions without the addition of PHA, or heat-killed lymphocytes (56° for 30 min) with 20 µg of PHA added at the time the cultures were established. After collection, the medium was concentrated twentyfold by ultrafiltration.

DEAE-Cellulose Column Chromatography. Previously washed Whatman DE-11 resin was poured into a 3.4 x 60-cm column and pressure packed (10 lb/in²) to a bed height of 30 cm. The columns were equilibrated with 10 mM Tris·HCl, (pH 8.0)-25 mM NaCl·0.1 mM EDTA buffer (low salt buffer) 100 ml (2 grams of protein) of concentrated toxic or control medium, previously dialyzed against low salt buffer and clarified by centrifugation, was applied to the column. Proteins were eluted from the column in a linear salt gradient from 25 mM to 350 mM NaCl in 10 mM Tris·HCl (pH 8.0)-0.1 mM EDTA buffer. 12-ml Fractions were collected, and the conductivity and absorbance at 280 nm of every fraction was measured.

Sephadex Chromatography. A 2.5 x 120-cm silicic acid-coated column (Chromatix, Berkeley, Calif.) was packed with Sephadex G-100 or G-150 to a bed height of 100 cm. Each column was pretested with molecular weight markers (blue dextran; molecular weight 2 x 10⁶), human IgG (150,000), bovine-serum albumin (67,000), and hemoglobin (68,000). Cytotoxic and control fractions from DEAE-cellulose columns were then pooled, lyophilized, solubilized in 5 ml of sterile distilled water, applied to the column, and eluted with 10 mM Tris·HCl (pH 8.0)-150 mM NaCl·0.1 mM EDTA buffer. 200-Drop (3.2-ml) fractions were collected, and their absorbance was monitored at 280 nm.

Density Gradient Centrifugation. Linear sucrose gradients (10-30%) were prepared in 5-ml cellulose nitrate tubes (11). Fractions from DEAE-cellulose columns (0.4 mg of protein) and 0.8 mg of bovine-serum albumin marker were layered on separate gradients and centrifuged at 35,000 rpm in a Beckman L3-40 ultracentrifuge for 45 hr. Absorbance at 280 nm of each of the 0.9-ml fractions was determined. The sucrose was removed by dialysis of the fractions against 4 liters of 10 mM Tris·HCl (pH 7.0)-150 mM NaCl·0.1 mM EDTA (physiologic dialysis buffer).

Abbreviations: LT, lymphotoxin; PHA, phytohemagglutinin.
been subjected were performed at the top of a glass column. A 0.5-ml sample was eluted with LT.

**Preparative Polyacrylamide Disc Electrophoresis.** Test and control DEAE-cellulose fractions were subjected to preparative polyacrylamide gel electrophoresis in our modification of the apparatus designed by Furlong et al. (15). A 6-cm layer of 7% polyacrylamide was first allowed to gel in a 1.5 x 12-cm glass column. Then, a 1-cm stacking gel was polymerized on top. A glass column of 1.5 x 2 cm was completely filled with a solution of 7% acrylamide. After polymerization, one end was covered with a piece of dialysis tubing. The short and long units were then joined together with a sleeve containing two elution ports, one on each side. 15 mg of LT containing the DEAE-cellulose fraction in 30% glycerol was added to the top of the column and covered with buffer. Electrophoresis was performed at 4° with a constant current of 7 mA in glycine buffer (pH 9), and fractions were eluted at a constant flow rate of one drop every 11 sec. 0.5-ml Fractions were collected, and absorbance was determined at 280 nm.

**Isoelectric Focusing.** These experiments were performed in 1% ampholine (pH 3-10) (16). Control and cytotoxic fractions were electrofocussed for 48 hr. After equilibration, 50-drop fractions (0.8 ml) were collected in an ISCO fraction collector at a constant flow rate of 1 drop per sec. The absorbance at 280 nm and pH of the fractions were measured, then fractions were dialyzed against 8 liters of physiologic dialysis buffer for 18 hr with four changes to remove the ampholine.

**Assay for Cytotoxicity.** After test and control media had been subjected to the various physical separation techniques, the residual buffers and salts were removed by dialysis against low salt buffer, physiologic dialysis buffer, or distilled water. Fractions dialyzed against hypotonic physiologic salt concentrations were reconstituted to physiologic tissue culture conditions by addition of a concentrated additive (9), or were added at 1:10 or 1:20 dilution directly to complete cell culture media. The medium was tested for cytotoxicity on cultures in tubes (17), or in microplates (18). Degree of cytotoxicity in microplates was determined by direct cell counts. In tubes, it was determined from the average of results of two different methods, a direct cell count made in a Coulter Counter, or the capacity of the remaining cells to incorporate [14C]laminoids into cellular protein after 48 hr of exposure to the test or control medium. The amount of LT activity in a sample was determined by testing the effect of doubling dilutions of test and control media on target cells. One unit of LT activity was defined as the amount that destroyed 50% of the target culture.

**RESULTS**

**DEAE-cellulose column chromatography**

Studies with protein-free and protein-containing media revealed that addition of bovine-serum albumin carrier (0.1%) to the initial culture medium helped protect the LT during the initial harvesting, storage, and concentration steps.

DEAE-cellulose is an ion-exchange resin that binds proteins with an overall negative charge. The proteins can then be differentially eluted from the column with an increasing gradient of cations. The location of LT activity after fractionation on DEAE-cellulose is shown in Fig. 1. LT activity eluted from the column with hemoglobin and before the bovine-serum albumin carrier. Fig. 1 is typical of over 30 separate column separations. When dilutions of the pooled LT-containing fractions were tested, 25–50% of the original activity was not recovered. A series of experiments in which all fractions eluted from the column were pooled and tested for activity revealed that this loss was irreversible. In later experiments, 20 mg of purified human hemoglobin was added to each sample to serve as a visible marker, because it was eluted with LT. Concentrated LT loaded on the column had from 1.5 to 2.5 units of activity per mg of protein. Pooled, concentrated fractions eluted from the column had from 35 to 110 units of activity per mg. Analysis of the DEAE-cellulose samples by acrylamide gel electrophoresis and immunodiffusion revealed 9–12 separate proteins. Control preparations had almost identical elution profiles, but little or no toxicity.
Sephadex column chromatography

Sephadex separates molecules according to their size, with the largest molecules eluting first. A typical Sephadex G-150 run containing LT with the marker proteins is shown in Fig. 2. The elution profile of LT from the columns was essentially the same, whether the original sample was crude medium or fractionated on DEAE-cellulose. This figure is representative of some 25–30 different column runs. LT activity eluted behind the IgG marker and in front of the bovine-serum albumin marker. When DEAE-cellulose fractions were applied to the column, LT fractions were collected with 320–1200 units of LT activity per mg of protein. Control runs had little or no toxicity. Recovery of LT activity was almost 100% in these columns. To see if LT was a small molecule, nuncovaletly linked to larger molecules, samples fractionated on DEAE-cellulose and crude samples were passed through the same columns in buffers containing various concentrations of NaCl, from 0.025 to 4.0 M. While there were various degrees of aggregation in 0.025 M and above 0.8 M NaCl, little or no aggregation occurred at 0.15 M NaCl. However, there was no major shift of the LT-elution pattern to regions containing smaller molecules during any of these studies. Analysis of the LT fractions on analytical polyacrylamide gels stained by Coomassie blue revealed several major and one minor protein bands.

Velocity sedimentation studies

Sedimentation of samples in sucrose gradients is a common method for obtaining an approximate value of the molecular weight of a macromolecule. DEAE-cellulose fractions were loaded on 5-ml linear 10–30% sucrose gradients. In each centrifuge run, one tube in the head contained a bovine-serum albumin marker, and the other tubes contained the LT samples. Typical results of these studies are shown in Fig. 3. It is clear that LT sediments just in front of the albumin marker.

Block electrophoresis

This is a method for separation or comparison of materials by observation of their migration in an electric field. The electrophoretic mobility of human LT was determined; the well-known migration pattern of human serum proteins was used as reference. 20-Times concentrated crude medium was used in both test and control separations. A typical result representative of nine separate tests is shown in Fig. 4. Each of the 280-nm absorbance peaks were identified in agar-slide immunoelectrophoresis, with rabbit antiserum directed against specific human serum proteins. LT activity migrated as a broad band, which localized in the region of the β- and α2 globulins. Although the total protein recovery was good, there was only a 25–50% recovery of LT activity. Moreover, when all fractions were pooled, concentrated, and tested, original activity was not regained.

Preparative polyacrylamide gel electrophoresis

This is a technique that simultaneously separates materials by charge and size. To further purify LT, DEAE-cellulose fractionated samples were separated on polyacrylamide gel columns. A typical result of one of these separations, containing phenol red, bovine-serum albumin and hemoglobin markers, is shown in Fig. 5. LT eluted behind, and was separable from, the hemoglobin marker. This is representative of 10 separate experiments. Recovery of LT ranges from 25–60%. In the most recent experiments, reduced glutathione (100 mM) was added; it improved recovery of LT activity by 15–25%. When DEAE-cellulose fractions containing LT were separated on the preparative column, they were found to contain very low concentrations of protein. Because of this, specific LT activity per mg of protein is difficult to calculate. However, it ranges from about 2000–4000 units/mg. Analytical polyacrylamide gels of concentrated LT fractions stained with Coomassie blue revealed one minor protein band in the area of LT activity in one of four experiments. However, these samples contain so little protein that we usually are unable to detect any bands.
Isoelectric focusing

Samples are electrophoresed in a sucrose and pH gradient until they reach their isoelectric point, when they cease to migrate. Resolution of closely related proteins is possible by this highly sensitive technique. Crude LT, DEAE-cellulose fractions, and samples that had been run through DEAE-cellulose and Sephadex G-150 columns were separated by isoelectric focusing. In all cases, LT activity was localized in the pH range between 6.8 and 8.0 in a pH gradient of 3-10. Control samples had little or no toxic activity. Final analyses of these fractions are, however, not yet completed.

DISCUSSION

We used two protein carriers, bovine-serum albumin, which stabilised human LT during the collection, storage, and concentration steps, and human hemoglobin, which served as a carrier and visible marker for LT through DEAE-cellulose and Sephadex.

Human LT is a macromolecule that has a molecular weight of about 85-100 × 10^6 or 3.2-4 S, as evidence by molecular sieving on Sephadex G-150 and velocity sedimentation through 10-30% sucrose gradients. The present studies suggest that it is not a small molecule complexed to a larger molecule by noncovalent bonds. Human LT is not a highly charged molecule, as evidenced by its early elution from DEAE-cellulose and slow migration rate in Pevikon and acrylamide gel electrophoresis.

Human LT is either a single molecule or several very closely related materials. We were unable to separate LT activity into multiple components. Also, the loss of activity seen in the various separations was attributed to inactivation, because it could not be recovered. However, there may be some heterogeneity among molecules with LT activity, because elution patterns were sometimes broad. This is supported by the broad spread of LT activity (pH 6.8-8.0) observed in isoelectric focusing experiments. Human LT is clearly separable from all carriers, serum proteins, and medium factors. With the combinations of DEAE-cellulose, Sephadex, and, finally, either the acrylamide gel electrophoresis or isoelectric focusing, degrees of purification in excess of 2000- to 4000-fold have been achieved, with 15-25% recovery of the original activity.

The role of factors released by lymphocytes in cell-mediated immune reactions in vivo and in vitro is still unclear. Purification and characterization of LT will greatly facilitate our ability to (a) determine the relationship of LT to the other lymphocyte effector molecules and (b) prepare specific blocking agents to provide the means whereby we can begin to elucidate the actual role assignable to this factor in cell-mediated reactions.

This research was supported by Grant AI 80490-3, from the Institute of Allergy and Infectious Diseases, NIH, and a grant from the Cancer Research Co-ordinating Committee of the University of California. G.A.G. is supported by a career award from N.I.A.I.D. of the NIH.