CELLULAR ORIGIN OF HUMAN LYMPHOTOXIN AND
ITS PURIFICATION

DISSERTATION

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The ability of various subsets of human mononuclear cells to produce human lymphotoxin (LT) was examined. Peripheral blood mononuclear cells were separated into OKT4⁺, OKT8⁺ and Leu-11a⁺ subpopulations by flow cytometry. Both OKT4⁺ and OKT8⁺ cells produced LT upon phytohemagglutinin (PHA) stimulation, but the OKT4⁺ T cells were the major source of LT. In contrast, Leu-11a⁺ cells failed to produce LT. The LT production and the proliferative response to PHA, of OKT4⁺ and OKT8⁺ cells, were inhibited by prostaglandin E₂ (PGE₂) and histamine. The LT, derived from PHA stimulated peripheral blood lymphocytes (PBL), was purified by a procedure involving Blue Agarose, Con A-Sepharose chromatography, preparative gradient polyacrylamide gel electrophoresis and preparative sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. The LT activity was recovered from SDS gel with major activity peak in the 76,000 Mr region and the minor activity peak in the 24,000 Mr region. The LT derived from 1788 lymphoblastoid cell line also showed heterogeneity on SDS gel. The activity was recovered from two peaks in the molecular weight regions of 70,000 and 20,000.

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CHAPTER I

INTRODUCTION

Lymphocytes, stimulated with mitogens, antigens and allogenic cells, release non-immunoglobulin soluble mediators. These mediators were designated as "lymphokines" by Dumonde in 1968 (10). They are produced by lymphocytes in minute quantities but have marked biological effects on a variety of cell types, including lymphocytes, monocytes, granulocytes, and fibroblasts. Although they were originally described as an in vitro phenomenon, recent evidence has shown that they can be also detected in vivo. It has become recognized that communication among T cells, B cells, monocytes and other cells in the immune system is mediated by the lymphokines, which act as communication signals between the cells producing them and the responding cells carrying the receptors for them.

Most lymphokines, if not all, are soluble glycoproteins of varying molecular weight (10,000 -100,000 daltons). In general, lymphokines tend to be classified as an antigen-specific or nonantigen-specific. Antigen-specific lymphokines react exclusively with the antigen which induced their formation. On the other hand, the nonantigen-specific lymphokines act in a polyclonal fashion. Over a hundred lymphokines have been discovered until now. They are characterized by their

biological activities in in vitro assays. Some exhibit more than one biological activity; this has resulted in giving the same factor different names.

Stimulated lymphocytes produce a variety of soluble mediators. One family of these mediators has been shown to exhibit cytotoxic and cytostatic effects on certain cell lines (24, 75). This mediator was later termed lymphotoxin (LT). As compared to most other lymphokines, the LT production occurs late (3 to 5 days after activation) (7). The LT can be produced by T cells, B cells, and null cells when appropriately stimulated (39, 74, 80, 89). Moreover, many lymphoblastoid cell lines carrying B cell surface markers can also release LT (3, 45, 71, 74). The LT molecule is complex; the first cell-lytic form released from PHA-stimulated T enriched lymphocytes is a 140-160,000 molecular weight specie which can be dissociated into smaller subclasses; α H, α , β , and γ LT (26).

The LT has been proposed as a mediator of T cell-mediated cytotoxicity. Indeed, there is both positive and negative evidence concerning its role in this reaction (27, 84, 92, 94). It has been reported by several investigators that LT exhibits selective cytolytic and cytostatic effects on tumor cell lines (52, 56, 60). Furthermore, it is able to inhibit chemical carcinogenesis and UV irradiation induced transformation (12). The antitumor activity of LT

has become recognized. Phase 1 clinical trial using LT containing preparations have been reported (9, 35, 72).

Both PGE₂ and histamine play an important role in the immune reaction. It has been well documented that PGE₂ and histamine suppress a variety of immune functions, including antibody production, T cell mediated cytotoxicity, and lymphokine production (8, 17, 18, 19, 55, 66, 68, 69, 70, 100). There are at least 2 theories to explain the suppressive mechanism of PGE₂ and histamine; (a) the compounds bind to specific receptors on lymphocytes and suppress the lymphocytes functions by raising intracellular cAMP (17, 46, 93); (b) the compounds directly activate the suppressor cells (8, 83, 95). The role of PGE₂ and histamine mediated immune suppression is an interesting issue in clinical immunology. Both compounds have been reported to be involved in immune deficiency in man (8, 18).

Although LT was discovered in 1968, the physiological role and the mechanism of action of LT are still not well defined. Further study is needed to clearly understand the role of this biologically active substance. The purposes of this research were to (1) determine the cellular origin of human LT; (2) study the suppressive effects of PGE₂ and histamine on a subset of lymphocytes; (3) improve the method of mass production of LT; and (4) purify and characterize the LT molecule.

CHAPTER II

LITERATURE REVIEW

Cellular Origin of LT

Many attempts have been made to identify the type of cells that produce LT. Treatment of murine lymphocytes with anti-theta serum and complement reduced the production of LT (80). This suggests that the T lymphocytes play a major role in the production of LT. Studies by Rosenau et al. (74) indicated that LT was produced by purified human T cells stimulated with a T cell mitogen, but not by purified human B cells stimulated with a B cell mitogen. In contrast, Klimpel et al. (3) were able to induce the production of LT from T cell-enriched populations and B cell-enriched populations by stimulation with appropriate mitogens. Other investigators also have demonstrated that human T cells, B cells and null cells, when appropriately stimulated, can produce LT (89). Moreover, LT can also be obtained from continuous cultures of lymphocytes carrying B cell surface markers (3, 23, 61, 74). The LT produced by these cell lines was found to be less heterogeneous (14, 64).

Using human T-enriched lymphocyte population, Harris et al. (26) demonstrated that the first cytolytic substance released from PHA stimulated cells was a large molecular

weight LT (140-160,000 daltons) which could be dissociated into small molecular weight subclasses, α H, α , β , γ LT upon chromatography on a molecular sieving column. The production of LT by subsets of mouse T cells has been investigated by Eardley et al. (11) who have shown that both Ly 1 (helper) cells and Ly 2 (cytotoxic/suppressor) cells sensitized with ovalbumin could produce LT; Ly 1 cells secreted more LT than Ly 2 cells. Additional studies are needed to determine if different types of LT are produced by different lymphoid cell subsets.

Mechanism of Action of LT

Little is known about the mechanism of action of LT. LT exerts a cytolytic action through binding with receptors on the membrane of the target cells. Correlation between high affinity binding of radio-labeled LT and sensitivity of target cells has been observed (88). The receptor for human LT has been shown to be sensitive to trypsin (28). Swada et al. (77, 78) have shown that several sugars having a terminal β -galactose as a terminal residue inhibit guinea pig LT activity. This suggests that the cellular receptor might have β -galactose as a terminal residue. A membrane glycoprotein which might serve as the LT receptor has been isolated (43, 50). Further characterization of the LT receptor no doubt will be reported in the near future.

Several studies have focused on the biochemical alterations in LT treated target cells. A marked increase in the CA^{+2} uptake by the guinea pig LT treated target cells has been observed (59). Rosenau et al. (73) found that human LT treated target cells do not show significant change in the K^{+} exchange, anaerobic or aerobic glycolysis, or ATP production. However, they consistently show an early increase in RNA synthesis, which includes changes in mRNA and is associated with an increase in nucleoplasmic RNA polymerase (47).

It has been suggested that LT causes target cell lysis by affecting the target cell membrane. Fried et al. (15) have shown by immunoelectron microscopy that the LT deposits are localized on the surface of the cell membrane. Weitzen and Granger (96) have demonstrated recently that a number of protease inhibitors inhibit the lysis of L929 (transformed mouse fibroblast) target cells by α LT. They have suggested that α LT is carried by the lymphocytes in an active form which can be activated by esterase to enable it to lyse the target cells. Membrane bound phospholipase A may also be activated after binding of LT to the target cells (42). The lytic mechanism of LT is very complex; one has to be cautious in drawing conclusions from many of the studies mentioned. Moreover, LT preparations also contain other biological active factors which can affect the target cell metabolism.

Biological Function of LT

The most debated issue among cellular immunologists is whether LT is the mediator of T cell mediated target cell lysis. Granger and his colleague (21) have initially proposed that target cell destruction by T cells is caused by LT, secreted by lymphocytes in response to antigenic or mitogenic stimulation. There are at least two approaches in studying the role of LT in cell-mediated cytotoxicity; a search for LT activity in cultures in which killer T cells have lysed the target cells, and the use of LT neutralizing antisera to block cell-mediated cytotoxicity. Many attempts have been made without success in detecting the LT activity in the killer cell reaction cultures. On the other hand, several investigations have been reported on the inhibition of different types of cytotoxicity reactions by the LT antisera. These include specific T cell killing (92, 94), lectin induced cellular cytotoxicity (30, 79), antibody dependent cellular cytotoxicity (45), and the natural killer cell function (97). The results of these studies have not been widely accepted and indeed, there are other pieces of evidence that do not favor the participation of LT in the cell-mediated cytotoxic events. It was found that the cytotoxic activity of lymphocytes could be readily dissociated experimentally from its ability to produce soluble mediator, including LT (27). Treatment of lymphocytes with a number of agents

(cholera enterotoxin, colchicin, and vinblastine) inhibited the direct lytic activity, but left unaffected the ability to produce LT. Alternatively, pectamycin and emitine treatment (10^{-7} to 10^{-6} M) suppressed production of LT, but had no effect on T cell mediated cytotoxicity (27, 84). Inhibition of LT activity was also observed upon exogeneous addition of Ca^{+2} in the range of 1 to 2 mM (59), a concentration that apparently showed no effect on T cell mediated-cytolysis (53, 65). The role of LT in T cell mediated-cytolysis has not been proved yet. There is both positive and negative evidence concerning this issue. There is clearly a need to define the role of LT in the cytolysis reactions. Monoclonal antibody against human LT is being produced in many laboratories. Using the monospecific antibody in the cytolysis neutralization experiment will certainly give a better picture of the role of LT in the cytolysis reaction.

Molecular Heterogeneity of LT

LT(s) are a group of proteins which can be separated from each other on the basis of their size, charge, and stability (20, 29, 31, 49, 91) (Fig. 1). Granger et al. (20) have reported a model of a subunit system of LT. The subunits are weakly lytic and can be associated to form a higher molecular weight complex, capable of rapid specific target cell lysis. The complex is unstable and rapidly dissociates into the smaller less effective components. The model has

Fig. 1. Size and charge heterogeneity of LT(s).
The LT(s) can be separated by gel filtration into various subclasses, including complex (Cx), alpha heavy(α H), alpha light(α L), beta (β), and gamma (γ) LT. The α H, α L, and β LT can be further separated by ion exchange chromatography.

been supported recently by Harris et al. (26). They have shown that the first cell-lytic form detected in PHA-stimulated T cells is a 140-160,000 molecular weight molecule. This molecule is converted into α H LT which can further dissociated into the small α , β , and γ LT forms.

A few studies have been done on the antigen specific LT. Hiserodt et al. (32) have shown that the high molecular weight complex but not the small form of LT is associated with an antigen binding receptor. The antigen-specific LT can bind specifically to the antigen immobilized on the column or adsorbed by the cellular antigen used for stimulation.

Assays for LT Activity

A variety of assays have been used to measure the cytotoxic activity of LT. Most of them have used a transformed mouse fibroblast cell line (L929) as the target cells.

L929 cells have been cloned and the most sensitive clone was selected for the assay. The sensitivity of the target cells can be further increased by treating them with mitomycin C (82). Because the assay is very important to purification studies and to study of the mechanism of action, several investigators have developed different methods of measuring the LT activity. These methods include:

(a) Labeled amino acid uptake assay (6, 22, 44)

(b) ^{86}Rb uptake assay (92)

- (c) ^{51}Cr release assay (62)
- (d) Microscopic counting assay (3)
- (e) Coulter counter enumeration assay (58, 76, 82)
- (f) ^3H Thymidine uptake, with computer aided analysis assay (81)

The disadvantages of these LT assays are the lack of sensitivity and accuracy, the time consuming nature and the use of radioactive materials. Recently, Khan et al. (37) developed an automated computer assisted microassay for LT. The target cells were plated into 96 well plates and incubated at 37°C over night. The next day, the LT samples were added to the wells and two-fold serial dilutions were made. After 18-24 hours of incubation, the viability of target cells was assessed by the uptake of neutral red dye using a Titertek Multiskan at 540 nm. The Multiskan was interfaced with the computer; therefore, the data from the Multiskan was directly transmitted to the computer and could be analyzed by a program written in Fortran 4. This assay was shown to have good accuracy, sensitivity and reproducibility and it was also less time consuming than others.

LT Purification

Purification of LT has been studied from a variety of supernatant culture sources such as human, guinea pig, rat, and mouse lymphocytes. Substantial purification of human LT was first described by Russell et al. (76). The LT

derived from PHA-stimulated adenoid lymphocytes was used for purification. The procedure involved gel filtration followed by DEAE chromatography and electrophoresis. The final product, purified approximately 2000 fold, had a molecular weight of 80,000 daltons. It was stable for several days at 4-22°C and stable for long period at -70°C. It was subsequently termed β LT by Walker et al. (91). At the same time, these investigators described a new form of LT which had a molecular weight of 45,000 daltons, was highly unstable and was produced early after PHA stimulation. Hiserodt et al. (29, 31) reported similar findings and were able to separate two species of LT by anionic exchange chromatography as well as by polyacrylamide gel electrophoresis. Recently, the radioiodinated alpha heavy and alpha light forms of LT have been purified to electrophoretic homogeneity (40, 41).

The purification of LT from established human lymphoid cell lines with the B cell surface markers has been reported (3, 14, 64). Further purification of LT from the cell line RPMI 1788 has been reported by Aggarwal et al. (1). The final product had a molecular weight of 20,000 daltons by SDS-PAGE under reducing conditions and had an isoelectric point of 5.8

Purification of LT from other animals has also been reported. Kolb and Granger (44) studied the purification of

mouse LT derived from lymphocyte stimulation by PHA-coated mouse L cells. The LT preparation was purified by ammonium sulfate precipitation and sephadex gel filtration. The final product had a molecular weight of 90-100,000 daltons. Additional work on the purification of mouse LT was reported by Trivers et al. (87). The molecular weight as determined by gel filtration was approximately 41,000 daltons, and had an isoelectric point of 4.4-4.8. In studies on guinea pig LT, Coyne et al. (6) reported the purification of LT derived from con A stimulated lymphocytes. The molecular weight by gel filtration was in the range of 35-50,000 daltons. The partially purified LT material was sensitive to chymotrypsin but not to neuraminidase. Further purification of guinea pig LT was reported by Gately et al. (16). The LT preparation was concentrated by ultrafiltration and purified by preparative Pelvikon block electrophoresis, DEAE chromatography, and polyacrylamide gel electrophoresis. The active fraction was re-electrophoresed and was found to contain a faint Coomassie blue staining band at the migration point of the cytotoxic activity. The final product had a specific activity of 300,000 units/mg of protein.

LT in Cancer Immunotherapy

Since the LT has been shown to have cytotoxic (24, 75) and cytostatic (25) effects with a degree of selectivity (52), many investigators have explored the use of LT for cancer

treatment. Papermaster and co-workers (61) have demonstrated that lymphokines from a continuous human lymphoid cell line (RPMI 1788) exhibit antitumor activity against human metastatic tumors. Intratumoral injection of the 10-50,000 molecular weight fraction from the RPMI 1788 cell line preparation in patients with disseminated malignant disease resulted in tumor regression in some patients (60). Khan et al. (36) have shown a regression of malignant melanoma in a dog by local injection of a human LT containing preparation. Phase 1 clinical trials using LT preparation derived from PHA-stimulated lymphocytes have also been reported (35). The only side effects observed in the study consisted of local pain, chills, and fever. Evidence of antitumor activity of LT is still too premature to judge.

The ability of LT to alter the carcinogenic process has been investigated. The anticarcinogenic activity of LT was initially recognized during a study of guinea pig cells during sequential stage of neoplastic transformation (13). The LT material purified by diafiltration and isoelectrofocusing was able to inhibit transformation as induced by chemical carcinogens or UV irradiation (12). The experimental use of LT for cancer treatment is in its early stages. The availability of pure LT material is now the major problem. This may be solved by producing LT through recombinant DNA technology. This type of work is currently underway in many laboratories.

CHAPTER III

MATERIALS AND METHODS

Target Cells

The L929 cells were obtained from the American Type Culture Collection. The monolayers were grown in RPMI 1640 (Gibco, Grand Island, NY.) containing 5% fetal bovine serum (Gibco), and 50 µg/ml gentamycin (Schering Corp., Kenilworth, NJ). The stock cultures were grown as monolayer in 75 cm³ tissue culture flasks (Costar, Cambridge, MA). The flasks were incubated at 37°C in 5% CO₂ atmosphere. The cell line was cloned using the limiting dilution technique; the most sensitive clone was used for the assay.

Lymphotoxin Assay

The L929-W cells were trypsinized from the stock cultures using phosphate buffered saline (PBS) containing 0.25% Difco trypsin and 0.2% EDTA. The cells were washed once in RPMI 1640 medium and then resuspended to a final concentration of 1×10^5 cells/ml. Mitomycin C (Sigma, St. Louis, MO) was added into the cell suspension at a final concentration of 0.5 µg/ml. One hundred and fifty µl/well of cell suspension was plated into 96 well plates (Costar) and incubated overnight at 37°C in 5% CO₂. The next day, the LT samples were

applied to the appropriate rows of the plates and two-fold serial dilutions were made. After 24 hours of further incubation, the supernatant fluid was aspirated from the wells followed by the addition of culture media containing 150 μ l/ml of neutral red at a volume of 150 μ l/well. The cells were incubated in the presence of dye for an hour and then washed with PBS. A hundred μ l of Sorenson's citrate solution (0.1M sodium citrate buffer, pH 4.2, and 95% ethanol in equal parts) was added to extract the dye. The plates were then read on the Titertek Multiskan at 540 nm. The data from the Multiskan were directly transmitted to the computer and analyzed by a program written in Fortran 4. The unit of LT activity was defined as the reciprocal of the highest dilution that caused 50% cytotoxicity as compared to the control.

Production of Human LT

Human leukocytes were obtained from buffy coats of normal blood bank donors (Wadley Central Blood Bank). The red blood cells were hemolysed with 0.83% NH_4Cl in Tris-HCl buffer (2.017M, pH 7.2). The white blood cells were washed twice with PBS and resuspended in RPMI 1640 medium containing 10% fetal bovine serum at a concentration of $3-4 \times 10^7$ cells/ml. The cell suspension was poured into a sterile nylon wool column (19 x 19 cm) and incubated for 45-60 minutes at 37°C in 5% CO_2 . The non-adherent cells were then eluted using pre-warmed (37°C) medium. These cells were then used to produce LT by incubating 1×10^6 cells/ml in RPMI 1640

containing 80 µg/ml of PHA-P (Difco, Detroit, MI), 1 g/ml of indomethacin (Sigma), 10^{-4} M cimetidine (Smith, Kline & French Lab, Carolina, PR), 20 ng/ml of 4-phorbol 12-myristate 13-acetate (TPA) (Sigma), 50 µg/ml gentamycin and 1% fetal bovine serum at 37°C for 5 days. At the end of the incubation, the cells were centrifuged at 1000g for 20 minutes, and the supernatant fluid was retained. The pool of supernatant fluids (15 liters) was concentrated and dialysed by the Pellicon System (Millipore, Bedford, MA) using a 10,000 Mr cut-off membrane. The crude material was kept frozen at -70°C until used.

Isolation of Subsets of Lymphocytes by Flow Cytometry

Human peripheral blood mononuclear cells were isolated from blood of healthy volunteers by Gicoll-hypaque (Pharmacia Fine Chemical, Piscataway, NJ) density gradient centrifugation (4). The monocytes were partially depleted by incubating the cell suspension in fetal bovine serum coated flasks for minutes at 37°C in 5% CO₂. The adherent cells were removed by gentle agitation with PBS containing 0.2% EDTA. The non-adherent mononuclear cells were then fractionated into OKT4⁺, OKT8⁺, and Leu-11a⁺ cells. In a typical experiment, 3×10^7 cells were labeled with fluorescein conjugated OKT4 or OKT8 monoclonal antibody (Ortho Diagnostic System, Raritan, NJ) or Leu-11a monoclonal antibody (Becton Dickinson, Mountain View, CA). The fluorescent cells were sorted and collected

with an Epic V cell sorter (Coulter Electronic, Hialeah, FL). Purity of the sorted cells was found by fluorescent microscopy to be in excess of 96%.

The Effect of Prostaglandin E₂ (PGE₂) and Histamine on the Proliferation of Cells and LT Production

The effects of PGE₂ and histamine on the subsets of T cells were measured. One hundred μ l/well of cell suspension, at a concentration of 4×10^5 cells/ml in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum was plated into 96 well plates. Both OKT4⁺ and OKT8⁺ cells were stimulated with 5 μ g/ml of PHA (Wellcome, Beckenham, England). The PGE₂ and histamine were also added into the culture at the same time as the PHA at a final concentration of 3×10^{-8} M and 5×10^{-4} M, respectively. To study the proliferative response of subsets of lymphocytes, the cells were pulsed with 0.5 μ Ci of ³H thymidine (New England Nuclear, Albany, MA), in 20 μ l of medium on day 3 and incubated for another 6 hours. The cells were then harvested on an automated cell harvester (Microbiological Associates, Walkersville, MD) and the ³H thymidine incorporation was determined (51). The remaining cultures were harvested on day 5 and LT levels in the supernatant were determined.

Blue Agarose Chromatography

Blue Agarose (Bethesda Research Lab, Bethesda, MD) was washed with a 10 column volume of 7 M urea to remove any dye

that might be non-covalently bound to the matrix. The column was then packed and equilibrated well with Tris-HCl buffer (0.01M, pH 7.2). The LT preparation containing a total activity of 1.2×10^6 units was pumped into the column at a rate of 10 ml/hr and the column was washed with 3 column volumes of Tris-HCl buffer. The LT activity was then eluted with Tris-HCl buffer containing 50% (v/v) ethylene glycol and 0.25 M KCl. Ten milliliter fractions were collected. The absorbance at 280 nm and the LT activity of each fraction was then measured. The LT containing fractions were pooled and stored at -70°C until used.

Con A-Sepharose Chromatography

The pool of LT containing fractions eluted from the Blue Agarose column was dialyzed against Tris-HCl buffer (0.01 M, pH 7.2) overnight. The LT material with a total activity of 7.8×10^5 units was applied to a Con A-Sepharose (Pharmacia) column at a rate of 10 ml/hr. The column was washed with 3 column volumes of Tris-HCl buffer. The bound LT was eluted with 0.4 M of α methyl-D-mannoside. Ten milliliter fractions were collected. The absorbance at 280 nm and the LT activity of each fraction was then measured. The active fractions were pooled and stored at -70°C for further purification.

Preparative Gradient Polyacrylamide Gel Electrophoresis

Horizontal polyacrylamide gel electrophoresis was performed on 10-40% gel. The slab gel (120 x 180 x 1.4 mm) was made using gel slab casting GSC-2 in conjunction with gradient mixer GM-1 (Pharmacia). The GM-1 was set up about 60 cm above the GSC-2 and the GM-1 was tilted by 10°, so that the outlet was at a lower level. The chamber nearest the outlet of the GM-1 was filled with a methanol solution (20% v/v in distilled water) and allowed to run down into the GSC-2, leaving the connecting tube filled with liquid and free of air bubbles. Then, the light gel stock solution (10%) was carefully poured into the lower chamber of the GM-1 and the heavy gel stock solution (40%) was poured into the other chamber. The stirrer was placed into the lower chamber and the paddle was turned on. The connecting valve between the two reservoirs and the tubing clip on the outlet tubing were opened simultaneously, allowing the solution to run into the cassettes. As soon as the gel solution had just run through, a heavy glycerol/water solution (1:1) was added to the lower chamber. The flow of the glycerol solution was stopped with the top of the polymerizing mixture had just reached the upper edge of the gel cassette. After the gel was completely polymerized, the slab gel was taken out from GSC-2 and laid on the cooling plate. Sample wells were cut 1 cm from the edge of the lower % gel. A dry powder of

Ultrodex (LKB, Rockvill, MD) was sprinkled into the wells to serve as sample support. LT with a total activity of 4.2×10^5 units was applied into the sample wells. Eight layers of Whatman No. 1 filter paper were used as the electrode. Horizontal gradient polyacrylamide gel electrophoresis was performed using the LKB Multiphore 2117. The electrophoresis was initially run at 25 mA for 45 minutes and then increased to 50 mA for 5 hours. Upon completion of electrophoresis, the slab gel was cut into 2-mm sections and the LT was eluted by homogenizing gel sections with a glass rod in 1 ml of Tris-HCl buffer (0.01 M, pH 7.2) containing 0.1% polyethylene glycol.

Preparative Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on linear 10-20% acrylamide gel containing 0.1% SDS. The slab gels were cast as described before. Purified LT material from preparative polyacrylamide gel electrophoresis was dialysed against Tris-HCl buffer (0.01 M, pH 8.3) and concentrated to 0.5 ml with Aquacide IIA (Calbiochem, La Jolla, CA). The concentrated material was incubated with SDS to give a final concentration of 0.1% (w/v) of detergent for 1.5 hours at 37°C and then applied into the sample well. The electrophoresis was

initially run at 25 mA for 45 minutes, and then increased to 50 mA for 5 hours. After the electrophoresis was complete, the slab gel was cut into 1-mm sections and the LT was eluted as described before.

CHAPTER IV

RESULTS

Cellular Origin of Human LT

Peripheral blood mononuclear cells were stained with fluorescein conjugated monoclonal antibodies and then fractionated with a fluorescence activated cell sorter (FACS) into T cell subsets (OKT4⁺ and OKT8⁺), and natural killer cells (Leu-11a⁺). In this study we used anti Leu-11a monoclonal antibody to stain the natural killer (NK) cells. Anti Leu-11a appeared to react with the Fc receptor on NK cells and did not react with T cell antigens (48, 63). Table 1 shows that OKT4⁺ and OKT8⁺ cells secreted different amounts of LT following stimulation with PHA. OKT4⁺ cells secreted approximately 14 times more LT than OKT8⁺ cells. The mixture of OKT4⁺ and OKT8⁺ cells produced intermediate amount of LT. In contrast, the Leu-11a⁺ cells, which have natural killer cell function failed to secrete significant amount of LT (<10 units/ml). The Leu-11a⁺ cells also showed poor response to PHA. Their uptake of ³H thymidine was one-tenth that of PHA stimulated PBL (data not shown). Many studies have shown that adherent cells inhibit lymphokine production (8, 19, 68).

This was confirmed since removal of adherent cells from PBL increased LT production and the addition of adherent cells led to reduce LT production by the OKT4⁺ and OKT8⁺ cells (Table 1).

Table 1

PRODUCTION OF LT BY DIFFERENT SUBPOPULATIONS
OF MONONUCLEAR CELLS*

CELLS	LT (UNITS/ML)
Mononuclear cells	284 $\pm$ 15
Non-adherent mononuclear cells	531 $\pm$ 11
OKT4 ⁺	3,102 $\pm$ 44
OKT4 ⁺ + adherent cells	1,450 $\pm$ 380
OKT8 ⁺	220 $\pm$ 113
OKT8 ⁺ + adherent cells	156 $\pm$ 83
OKT4 ⁺ + OKT8 ⁺	900 $\pm$ 230
Leu-11a ⁺	<10

*One hundred μ l of each sub-population at a concentration of 4×10^4 cells/well was seeded into 96 well plate. In the combination study, the adherent cells were added into each cell culture at a concentration of 4×10^3 cells/well in addition to the 4×10^4 lymphocytes. In the wells containing the mixture of OKT4⁺ and OKT8⁺ cells, 2×10^4 cells of each type were added into each well. The cultures were stimulated with 5 μ g/ml of PHA (Wellcome) for 5 days and LT production was measured.

Kinetics of LT Production

The difference in kinetics of LT production between unfractionated peripheral blood lymphocytes and T-enriched peripheral blood lymphocytes has been recently reported (26). We examined the possibility that different subsets of T cells may have different patterns of production. The results are shown in Figure 2. Unfractionated PBL, OKT4⁺ and OKT8⁺ cells appear to have the same kinetics of LT production. The activity reached its peak after 4 days with no further change up to day 6.

Inhibitory Effects of PGE₂ and Histamine on LT Production

Both PGE₂ and histamine have been shown to have inhibitory effect on a variety of lymphocyte functions including mitogen- or antigen-induced cell proliferation, cytolysis, antibody production and lymphokine production (8, 17, 18, 19, 55, 68, 69, 70, 71, 100). Sorted cells were stimulated with PHA in the presence of PGE₂ or histamine. Both subsets, OKT4⁺ and OKT8⁺ cells were suppressed by PGE₂ and histamine. Prostaglandin₂ (3×10^{-8} M) suppressed the proliferative response of OKT4⁺ and OKT8⁺ cells by $26 \pm 17\%$ and $27 \pm 18\%$. Histamine (5×10^{-4} M) suppressed the response of OKT4⁺ and OKT8⁺ cells by $52 \pm 16\%$ and $32 \pm 19\%$ respectively (Fig. 3a). The ability of OKT4⁺ and OKT8⁺ cells to produce LT in the presence of PGE₂ and histamine was also investigated. The results indicate

that PGE_2 inhibited LT production in OKT4^+ and OKT8^+ cells by $63 \pm 19\%$ and $82 \pm 6\%$ respectively whereas histamine showed $43 \pm 5\%$ and $66 \pm 8\%$ inhibition respectively (Fig. 3b).

Improvement of LT Production

Several methods were used to remove the adherent cells from the buffy coat cells. The nylon wool column was found to be more effective than glass bead or sephadex G-10. The passage of buffy coat cells through a nylon wool column resulted in enrichment of lymphocytes. The percentage of lymphocytes in the nylon wool non-adherent cell fraction was found to be approximately 2.5 times that in originally buffy coat cells (Table 2 and Fig. 4). Analysis of lymphocyte subsets showed no loss of T cells on the nylon wool column, but some B cells (56%) adhered to the nylon wool column (Table 2). Upon PHA-stimulation, nylon wool non-adherent cells produced larger amounts of LT than did that unfractionated buffy coat cells. The addition of indomethacin, cimetidine, and 4 β -phorbol 12-myristate 13-acetate (TPA) further enhanced LT production (Table 3).

Purification of LT

The crude concentrate of LT with a 10,000 Mr cut-off was first applied on a Blue Agarose column. The LT was successfully eluted with 50% (v/v) ethylene glycol, 0.25 M KCl in Tris-HCl buffer (0.01 M, pH 7.2) (Fig. 5). Albumin

constituted a major contaminant at this stage. The material from Blue Agarose was then applied to Con A-Sepharose column. The LT activity was eluted with 0.4 M α -methyl-D-mannoside in Tris-HCl buffer (Fig. 6). A small residual LT was consistently found in the unbound fraction of both preceding columns.

TABLE 2
SURFACE MARKER ANALYSIS OF CELL FRACTIONS
OBTAINED FROM BEFORE AND AFTER PASSAGE
THROUGH A NYLON WOOL COLUMN*

Steps	% Lymphocytes	% OKT4 ⁺ Cells	% OKT8 ⁺ Cells	% Surface Immunoglobulin Positive Cells
Before nylon wool column	27 $\pm$ 5	41 $\pm$ 1	24 $\pm$ 4	14 $\pm$ 8
After nylon wool column	67 $\pm$ 8	47 $\pm$ 3	29 $\pm$ 5	6 $\pm$ 5

*The cell fractions obtained from before and after passage through a nylon wool column were stained with fluorescein-conjugated OKT4 or OKT8 monoclonal antibody or (Fab)₂ of goat anti-human immunoglobulin. The percentage of OKT4⁺ cells, OKT8⁺ cells and surface immunoglobulin positive cells in the lymphocytes fraction were analyzed by Epic V. The percentage of lymphocytes was analyzed from Wright-Giemsa-stained cytocentrifuge preparations.

The final two steps of purification involved electrophoresis on a gradient polyacrylamide gel and SDS polyacrylamide gel. On the preparative gradient polyacrylamide gel electrophoresis, the LT activity comigrated with a protein band (Fig. 7) and most of the contaminating proteins were eliminated. Lymphotoxin eluted from gradient gel was then

applied to SDS-PAGE. The major LT activity corresponded to Mr 76,000 and a minor peak corresponded to Mr 24,000 (Fig. 8). The two activities were reapplied to SDS-PAGE. The activity again matched with the 76,000 Mr protein band (Fig. 9). The 24,000 Mr fraction showed a single protein band, but we did not have enough material to check for activity.

TABLE 3
IMPROVEMENT OF LT PRODUCTION*

Experiment Groups	LT (units/ml)
Buffy coat cells	57 $\pm$ 15
Nylon wool non-adherent cells	255 $\pm$ 130
Nylon wool non-adherent cells + Indomethacin (1 μ g/ml) + Cimetidine (1 x 10 ⁻⁴ M)	370 $\pm$ 154
Nylon wool non-adherent cells + Indomethacin (1 μ g/ml) + Cimetidine (1 x 10 ⁻⁴ M) + TPA (20 ng/ml)	940 $\pm$ 124

*The cells from each group were incubated at 1 x 10⁶ cells/ml in RPMI 1640 medium containing 80 μ g/ml of PHA (Difco) and 5% fetal bovine serum at 37°C for 5 days. At the end of incubation, the LT activity was then measured.

We also studied the LT from the lymphoblastoid cell line RPMI 1788. The LT was purified by Con A-Sepharose chromatography, isoelectrofocusing and gradient polyacrylamide gel electrophoresis. The final material was then analyzed on

SDS-PAGE. Interestingly, the two active species recovered were located at 70,000 and 20,000 Mr respectively (Fig. 10).

Fig. 2. Kinetics of production of human LT from unfractionated PBL, OKT4⁺ cells and OKT8⁺ cells. Each population was cultured in RPMI 1640 containing 10% heat inactivated fetal bovine serum at a cell density of 4×10^5 cells/ml. The cell cultures were then stimulated with 5 μ g/ml of PHA. Samples were removed at various times and the activity was then measured. Each point represents the mean $\pm$ S.E. for three experiments (■—■ PBL, ▲—▲ OKT4⁺ cells, ●—● OKT8⁺ cells).

Fig. 3. Inhibition of ^3H thymidine incorporation and LT production by PGE_2 and histamine. The cells of each population were cultured in RPMI 1640 containing 10% heat inactivated fetal bovine serum. The cell cultures were stimulated with 5 $\mu\text{g}/\text{ml}$ of PHA. Prostaglandin or histamine was also added to the cultures at the same time as PHA at a final concentration of 3×10^{-8} M and 5×10^{-4} M, respectively. After 3 days, the ^3H thymidine incorporation was determined (a). The remaining cultures were harvested on day 5 and LT production was determined (b). Each bar represents the mean value $\pm$ S.E. of three experiments (■ PGE_2 , □ histamine).

Fig. 4. Light scatter histogram of the cell fraction before and after passage through the nylon wool column. Fifty thousand cells of each fraction were analyzed by Epic V (Coulter Electronic) and two histograms comparison were made. In this particular experiment, the cells which occupied in the lymphocyte channel was increased from 31 to 74% after passing the buffy coat cells over the nylon wool column.

Fig. 5. Blue Agarose chromatography of LT. The crude material of LT was applied to Blue Agarose column. The bound proteins were eluted with 50% (v/v) ethylene glycol, 0.25 M KCl in Tris-HCl buffer (0.01 M, pH 7.2). The LT activity (●—●) and protein concentration (●—●) of each fraction was measured.

Fig. 6. Con A-Sepharose chromatography of LT. The LT fraction from the Blue Agarose column were pooled, dialyzed against Tris-HCl buffer (0.01 M, pH 7.2) and then applied to Con A-Sepharose column. The bound proteins were eluted with 0.4 M α -methyl-D-mannoside in Tris-HCl buffer (0.01 M, pH 7.2). The LT activity (●—●) and protein concentration (●—●) of each fraction was measured.

Fig. 7. Preparative gradient polyacrylamide gel electrophoresis (10-40%) of LT. The partially purified LT from Con A-Sepharose column was electrophoresed in 10-40% gradient gel. After the run was completed, a small part of the gel was stained with Bio-Rad silver stain as a reference. The rest of the slab gel was sliced into 2-mm fractions and the proteins were eluted. The LT activity of each fraction was measured (●—●).

Fig. 8. Preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis of LT. The purified LT material from gradient polyacrylamide gel electrophoresis was treated with SDS at a final concentration of 0.1% for 1.5 hours. The treated sample was then applied to a sample well. After electrophoresis was completed, the gel was sliced into 1-mm fractions. The LT was eluted and checked for activity (—○—).

Fig. 9. Analytical sodium dodecyl sulfate polyacrylamide gel electrophoresis of LT. Purified LT extracted from 76,000 Mr region (13,000 units) and from 24,000 Mr region (350 units) from preparative SDS-PAGE were re-electrophoresed on a SDS gel. For the 76,000 Mr species, 10,000 units was applied and stained as a reference. Another 3,000 units were run site by site for assay of LT activity. The proteins were stained with the silver stain. (A) purified LT extracted from 76,000 Mr region; (b) crude LT; (c) protein standard; (D) purified LT extracted from 24,000 Mr region.

Fig. 10. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of LT from cell line 1788. The LT from 1788 cell line was purified by Con A-Sepharose chromatography, isoelectrofocusing, and gradient polyacrylamide gel electrophoresis. The final materials were analyzed on SDS-PAGE. After electrophoresis was complete, the gel was sliced into 1 mm fractions. The LT was eluted and checked for activity ( ).

Table 4
PURIFICATION OF HUMAN LT

STEPS	PROTEIN (mg)	TOTAL ACTIVITY UNITS	SPECIFIC ACTIVITY UNITS/mg	YIELD (%)
Starting Material	3200	1.2×10^6	375	100
Blue Agarose	192	7.8×10^5	4100	65
Con A-Sepharose	5	4.2×10^5	84000	35
Preparative Gradient Gel Electrophoresis	0.116	8.4×10^4	720000	7
Preparative SDS gel Electrophoresis	N.D.	1.3×10^4	N.D.	1

N.D. = not done

CHAPTER V

DISCUSSION

During the past two decades of work on LT, a great deal has been learned about this molecule, including its production, the physiocochemical properties, and its cytolytic mechanism. But the precise role of LT in mediating the immune reaction is still unknown. There is a need to prove conclusively whether the LT molecule represents the cytotoxic mediator released by effector cells and causes the target cell's death.

The logical approach towards determining LT's function would be to characterize the cells that produce it, since certain physiological roles in the immune response have been assigned to the cells expressing a unique pattern of cell surface markers. The human helper T cell antigen marker can be recognized by monoclonal antibody OKT₄, whereas the human cytotoxic-suppressor T cell antigen marker can be recognized by monoclonal antibody OKT8 (46,47).

Here, we report the cellular origin of human LT. By fractionating mononuclear cells into subsets of OKT₄⁺, OKT8[±] and Leu-11a⁺ cells with a fluorescence activated cell sorter (FACS), we have shown that both helper as well as cytotoxic/suppressor T cells produce LT. The OKT₄⁺ cells (helper)

were found to secrete more LT than OKT8⁺ cells (cytotoxic/suppressor). The Leu-11a⁺ cells which have natural killer cell function, showed no response to PHA and did not secrete a significant quantity of LT upon PHA stimulation. A subpopulation of natural killer cells which were recognized by HNK -1 monoclonal antibody also were unresponsive to mitogens (PHA, pokweed mitogen (PWM), concanavalin A (Con A) or to allogeneic cells (86). On the other hand, Kasahara et al. (34) recently reported that large granular lymphocytes (LGL) were able to produce multiple lymphokines in response to PHA or Con A. The cells which showed OKT3⁻, Leu 1⁻ and OKT11⁺ cells appeared to be the source of the LGL derived interleukin 2 (IL₂). The difference in the findings may be explained on the basis of the heterogeneity of LGL. The subpopulation of LGL defined as Leu-11a⁺ in our hands, exhibited no response to PHA and did not produce lymphokines. It is possible that Leu-11a monoclonal antibody may suppress the response of the cells to PHA.

There was no difference in the pattern of LT production kinetics among unfractionated peripheral blood mononuclear cells and T cell subsets. The LT production reached its peak after 4 days and the level remained unchanged until day 6. Harris et al. (26) have reported different production kinetics. PHA stimulated T enriched human PBL were shown to reach the peak of LT production at 46 hours. A major difference in the

two studies was the procedure used for cell fractionation. We used the cell sorter for this purpose while Harris and coworkers used SRBC rosettes to isolate T cells, and the rosetting cells were cultured without the removal of SRBC's.

The role of LT in cell-mediated cytotoxic reaction is unclear. Our data suggested that OKT4⁺ cells were the major source of LT. It has been shown that the cytotoxic effector cells, generated from PHA-activated PBL, were OKT8⁺ cells. However, it has also been reported that OKT4⁺ cells can also function as cytotoxic effector cells (33, 57). Furthermore, purified OKT4⁺ cells when sensitized with alloantigen, show more cytotoxic capacity than OKT4⁺ cells (67). These reports, therefore, suggest that, after proper stimulation, OKT4⁺ cells can act as cytotoxic cells and the interaction of these cells or their products with OKT8⁺ cells leads to the generation of OKT8⁺ cytotoxic cells (67).

The role of cytotoxic mediator in the natural killer cell mediated target cell lysis has been investigated. Wright et al. (99) reported that co-cultured human peripheral blood lymphocytes with NK-sensitive or resistant cell lines stimulated the release of the natural killer cytotoxic factor(s) (NKCF), which was selectively cytotoxic to NK-sensitive target cells. The factor(s) was nondialyzable, stable at 56°C for 3 hours, and showed partial loss of activity on storage at 4°C or -20°C in 7 days. Recently, Weitzen et al. (97) have shown

that rabbit anti human LT antisera inhibited human NK-induced cell lysis. The data indicate that some form of LT may be involved as an effector in NK activity. Our data have shown that Leu-11a⁺ cells, which have NK cell function did not produce LT upon PHA stimulation. It would be interesting to determine if Leu-11a⁺ cells produce any cytotoxic factor upon co-culture with NK target cells.

Several studies have been directed at the mechanism of PGE₂ and histamine induced suppression of lymphocyte function. Some studies have suggested that both PGE₂ and histamine bind to specific receptors on lymphocytes and suppress the lymphocyte function by raising intracellular cAMP. Following a rise of cAMP in lymphoid cells, metabolic functions such as glucose utilization, RNA and protein synthesis and other functions are impaired (17, 66, 93). However, the suppressive effect of PGE₂ and histamine is now thought to be mediated through the activation of the suppressor cells. Webb and Nowowiejski (95) reported that PGE₂ did not directly suppress the proliferative response of mouse splenocytes to mitogens. Instead PGE₂ activated glass-wool-adherent T cells, which released a low molecular weight peptide that highly suppressed both T and B cells. Prostaglandin was able to suppress proliferative responses of splenocytes only in the presence of these glass-adherent-suppressor T cells. In man, Chouaib and Fradelizi (5) found no inhibition of IL₂

production by PGE_2 if the T cell suspension was irradiated before the addition of PGE_2 or of excess monocytes. They suggested that activation of radiosensitive T cells by PGE_2 is required for the inhibition of IL_2 production. In this study, we have shown that the proliferative response and LT production by OKT4^+ and OKT8^+ cells were suppressed by both PGE_2 and histamine. The finding that purified OKT4^+ cells (helper T cells) were suppressed by PGE_2 and histamine indicates that these compounds do not suppress lymphocytes solely through activation of suppressor cells. Our observation suggests that both PGE_2 and histamine at the concentrations employed appeared to have a direct effect on the lymphoid cells and inhibited their cell functions. Tilden and Bach (85) have reported similar results. They found PGE_2 to be equally suppressive for OKT8^+ and OKT8^- cells, and to inhibit the action of IL_2 as well as its production. The immunosuppressive effects mediated by PGE_2 and histamine are a very important issue from the clinical point of view. Further studies are needed to clarify the suppressive mechanism of both compounds.

We have studied the method for improvement in the mass production of LT. The adherent cells were removed by passing the cell suspension over a nylon wool column. Removal of adherent cells was necessary for optimal production of LT. The adherent monocytes are the main producers of PGE_2 which

can inhibit lymphokine production (17). It has been suggested that the lymphokines released by lymphocytes can activate macrophage to produce PGE_2 which then inhibits the lymphocyte functions (17). Addition of indomethacin (cyclooxygenase inhibitor) and cinetidine (H_2 -receptor antagonist) to the lymphocyte culture caused further increase of LT production. TPA which had been shown to increase the gamma IF (90), also increased the LT production in our study. It is possible that TPA enhance the lymphokine production by binding to the receptors on the cell surface and delivers the second message to increase the rate of transcription and translation.

Purification of human LT has been reported by several investigators in the past (22, 76, 64). Radioiodinated alpha light and alpha heavy forms of LT have been purified to electrophoretic homogeneity on PAGE (40, 41). The progress of LT purification has been limited mainly due to the small quantity of starting material. In this study we have improved the purification by introducing preparative electrophoresis, including gradient polyacrylamide gel electrophoresis and SDS-PAGE. Preparative isoelectrofocusing was discarded due to unacceptable loss of activity. The LT activity was recovered was very low (1%) (Table 4). Two peaks of LT activity were recovered from SDS-PAGE under non-reducing conditions with the major activity peak corresponding to

24,000 Mr Heterogeneity of LT has been well demonstrated (20, 29, 31, 49, 91). It is possible that the native form of LT is an oligomer, which provides more lytic activity and stability than the monomeric form. The 76,000 Mr LT may represent the undissociated native form, and 24,000 Mr may represent a monomeric form which dissociated by 0.1% SDS treatment.

Similar heterogeneity of LT from a lymphoblastoid (1788) cell line was seen on SDS-PAGE under non-reducing conditions. From this preparation, two activity peaks were recovered which corresponded to 70,000 Mr and 20,000 Mr. Recently, Aggarawal, et al. (1) reported the purification to homogeneity of LT from the 1788 cell line. The final product showed a single protein band at a molecular weight of 20,000 Mr on SDS-PAGE under reducing conditions. The higher molecular weight form of LT, which was detected in our studies, is likely to represent the aggregate form.

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