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Journal
Cellular Immunology, 24(2)

ISSN
0008-8749

Authors
Hiserodt, John C
Prieur, Anne-Marie
Granger, Gale A

Publication Date
1976-06-01

DOI
10.1016/0008-8749(76)90212-4

CELLULAR IMMUNOLOGY 24, 277–288 (1976)

In Vitro Lymphocyte Cytotoxicity

I. Evidence of Multiple Cytotoxic Molecules Secreted by Mitogen Activated Human Lymphoid Cells *in Vitro* *

JOHN C. HISERODT,[1] ANNE-MARIE PRIEUR,[2] AND GALE A. GRANGER[1]

[1] *Department of Molecular Biology and Biochemistry, University of California, Irvine, Irvine, California 92717, and* [2] *Clinique Medicale des Enfants, Hopital des Enfants Malades, 149, Rue de Sevres, Paris (XI^e), France*

Received February 3, 1976

Multiple families of cytotoxic molecules [Lymphotoxin (LT)] have been identified in phytohemagglutinin (PHA-P) activated human lymphocyte supernatants and lymphocyte homogenates, using gel filtration chromatography on Sephadex G-150. These macromolecules have molecular weights of 80–90,000, 50,000, and 10–15,000 daltons and have been termed LT_1, LT_2 and LT_3, respectively. They are secreted by cells from a variety of lymphoid tissues, *i.e.*, tonsil, adenoid, and peripheral blood. The kinetics of appearance of the cytotoxins indicate that all three are present within 16 hr after lymphocyte activation. However, while LT_1 and LT_2 persist in these cultures through day 5, LT_3 is not detectable after day 3. These molecules can also be detected when either PHA or concanavalin A are employed as the stimulating agent. Moreover, the relative amounts of LT_1, LT_2 and LT_3 activity in a given supernatant vary dramatically from culture to culture. Extracellular levels of LT accumulate and peak by 4 to 5 days in culture, however, intracellular levels of LT reach a maximum on day 3 and decrease to very low levels on day 5. Mitogen-stimulated lymphocytes at 3 days contain intracellular levels of LT which are several logs higher than that detectable in unstimulated cells. This observation suggests that both the biosynthesis and secretion of lymphotoxin is governed by a regulatory control process(es).

INTRODUCTION

A variety of biologically active materials, termed lymphocyte effector molecules (LEM),[3] are released *in vitro* when lymphoid cells are activated nonspecifically with mitogens, specifically with antigens, during mixed lymphocyte reactions, or from cultures of continuous lymphoid cell lines (1, 2, 3). These substances exhibit a wide spectrum of biologic activities. One such activity termed lymphotoxin (LT) (4, 5, 6) is cytostatic or cytotoxic to nonlymphoid cells *in vitro* (7). It has been

* This research was supported by Grant AI-09460, from the Institute of Allergy and Infectious Diseases, NIH, Grant No. IM-32, from the American Cancer Society, Grant No. 1883, from the Rheumatic Diseases Research Foundation, and Grant No. 5 KO4 GM 25607, from the Career Development Branch of the NIH.

[3] Abbreviations used: PHA-P, phytohemagglutinin-P; Con A, concanavalin A; SAL, supernatant from activated lymphocytes; LT, lymphotoxin; PBS, phosphate-buffered saline; FCS, fetal calf serum; NBCS, newborn calf serum; BSA, bovine-serum albumin; LEM, lymphocyte effector molecules.

suggested to be the active component involved in cellular immune tissue destructive reactions, such as tumor immunity and allograft rejection (1). The activities associated with LT have suggested that it is a relatively heat stable, trypsin sensitive protein(s) whose physico-chemical properties are currently under much investigation.

There are numerous reports as to the nature of material(s) with human LT activity derived from mitogen stimulated lymphoid cells or continuous human lymphoid cell lines. The majority of investigators find a proteinaceous molecule of 90,000 MW with an electrophoretic migration similar to a slow beta or fast serum gamma globulin (1, 8–11). However, several investigators have reported multiple peaks of cytolytic activity detectable in these same supernatants (12–14). The present manuscript supports the latter observations and further defines the existence of multiple members of the intracellular and secreted LT family of human lymphocyte effector molecules.

MATERIALS AND METHODS

Target cells and culture media. Stock cultures of mouse alpha L-929 fibroblasts were used as target cells (15). These cells were maintained in 32 oz prescription bottles in 95% air, 5% CO_2 and passed biweekly. Culture media consisted of minimal essential media with Hanks salts, supplemented with 3% heat inactivated (56°, 60′) fetal calf serum (Microbiological Associates, Bethesda, Md). 0.2 μg/ml glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) (MEMS).

Lymphocyte cultures. Suspensions of human small lymphocytes from tonsils or adenoids were obtained within 6–8 hr of surgical removal from normal children, as previously described (16). Lymphocyte suspensions were adjusted to 4×10^6 viable cells/ml in 32 oz prescription bottles using MEMS and incubated for either 1, 3 or 5 days in 95% air, 5% CO_2 at 37°C. Activation of the lymphocytes was effected by the addition of phytohemagglutinin-P (PHA-P, Difco Laboratories, Detroit, Mich.) at 20 μg/ml or concanavalin A (Sigma, St. Louis, Mo.) at 2 μg/ml.

Lymphocytes from defibrinated freshly drawn peripheral blood were obtained by gravity sedimentation in 3% gelatin (Sigma, St. Louis, Mo.) at 37°C. The lymphoid cells from this procedure were then further purified on a density gradient using ficoll-hypaque. Microscopic observation routinely showed 90–95% lymphocytes having 95–100% viability by Eosin Y dye exclusion. Cultures of peripheral blood lymphocytes were set up identical to those for tonsils or adenoids.

Lymphocyte supernatants. Culture supernatants were collected by centrifugation at 400*g* for 10 min, followed by filtration through a 0.45 μ millipore filter. Supernatants were stored at −70°C until used. Crude supernatants were concentrated by ultrafiltration on Amicon filters (Amicon Corp., Lexington, Mass.) using a PM10 membrane. This retains all materials having molecular weights greater than 10,000 daltons. Day 1 supernatants were routinely concentrated 20 to 40×, day 3—10 to 20×, and day 5—less than 10×.

Cytotoxicity assays. Lymphotoxin (LT) was assayed in crude or concentrated supernatants by determining the percent survival of mitomycin C treated target cells as previously reported (17). Briefly, L cells were established as monolayers in screw capped tubes at a concentration of 10^5 cells in 1.0 ml in the presence of 0.5 μg/ml mitomycin C (Sigma, St. Louis, Mo.). Following overnight incubation at

37°C, the supernatants were decanted, the monolayers washed with phosphate buffered saline [0.15 *M* NaCl, 0.01 *M* phosphate, pH 7.2 (PBS)], and serial dilutions of LT or control medium in MEMS were added. The tubes were then incubated for 24 hr at 37°C or 34°C and the dead cells which had detached from the monolayers poured off in the supernatants and the remaining adherent cells removed by trypsin treatment. These trypsinized cells were then enumerated on a Model F Coulter Counter. A unit of LT is defined as that amount necessary to cause a 50% destruction of 10^5 viable cells following a 24 hr incubation. The amount of LT activity in units/ml in a given supernatant is determined by testing serial dilutions of medium and obtaining the reciprocal of the last dilution killing 50% of the target cells.

Sephadex column fractions were filter sterilized by passage through a 0.45 μ millipore filter and assayed for LT by adding 0.10 or 0.20 ml to 1 ml of MEMS on preestablished tube cultures of 10^5 L cells. After a 24 hr incubation at 37°C, the viable adherent cell number was determined on a Coulter Counter.

Gel filtration chromatography. A 2.5 × 120 cm siliclad coated column (Chromatronix, Berkeley, Calif.) was packed with Sephadex G-150 to a bed height of 100 cm. The columns were equilibrated with PBS pH 7.2 containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml fungizone (PBS/SPF). Calibration was effected using various molecular weight markers: Blue dextran (2×10^6 daltons), bovine serum albumin (68,000), ovalbumin (44,000), myoglobin (17,000), cytochrome C (13,500), and phenol red (354 daltons). These markers were applied to the bottom of the column in a total volume of 2.0 ml and eluted with reverse flow in PBS/SPF. The flow rate was adjusted to 25 ml/hr and fractions were collected in 4.5 ml aliquots. The absorbance of each fraction was monitored at 280 nm with a Gilford Model 2000 spectrophotometer.

Whole cell disruption. Homogenates of whole cells were obtained in the following manner. Either alpha L cells or PHA stimulated lymphocytes were washed with PBS containing 1% newborn calf serum and resuspended in 8 to 10 ml of MEM. They were then sonicated for 4 min at 25°C, using a Bransonic 32 sonicator. HeLa cells and unstimulated lymphocytes were also disrupted using a Vertis Ti 60 at 10,000 rpm for 5 min. After complete lysis, verified by microscopic observation, the homogenates were centrifuged, 10,000*g*, 15 min, and the supernatants filter sterilized by passage through a 0.45 μ millipore filter.

RESULTS

Resolution of Multiple Human LT Activities in Supernatants of Activated Lymphocytes (SAL) by Gel Filtration Chromatography

Gel filtration chromatography employing sephadex is a powerful biochemical technique for separating macromolecules according to size, with the largest molecules eluting first. The elution profile of various MW markers applied to a 2.5 × 100 cm Sephadex G-150 column is shown in Fig. 1A. Each new column was calibrated by the same methods and in all separation profiles shown, the locations of the various markers are illustrated by elution bars.

As reported previously, we noticed that chromatography of 5.0 ml of concentrated SAL from PHA-stimulated cultures of human adenoid cells showed LT activity eluting as a wide peak just in front of the BSA marker (9–11). However,

when smaller volumes, *i.e.*, 1 to 2 ml of these same SAL supernatants were chromatographed, multiple peaks of LT activity were observed. Shown in Fig. 1B is the elution profile of a 2.0 ml 25× concentrated SAL obtained 16 hr after PHA stimulation. The first peak of LT activity appears just in front of the BSA marker at a MW of about 80–90,000 daltons. However, a second peak was observed which elutes with the ovalbumin marker indicating a MW of approximately 50,000 daltons. In addition, a new peak of activity was detected eluting from the column just behind the myoglobin marker, indicating a size between 10,000 and 15,000 daltons. To facilitate further studies, we termed these peaks LT_1, LT_2 and LT_3, respectively. These results have been repeated in numerous experiments. We have also noticed on occasion, during chromatography of other SAL, variable levels of toxic activity eluting in the void volume with the blue dextran marker. We believe this activity to be due to LT aggregates, as were similarly seen by Boulos, *et al.* (12). In contrast, Fig. 1A shows that chromatography of 30× concentrated control media containing PHA and FCS showed no toxic peaks, as previously reported (8, 18). It is important to note that maximum resolution of these multiple LT activities will be obtained by: 1) testing column fractions at the correct concentration in the bioassay, and 2) only applying small volumes to the column to minimize peak overlap due to diffusion.

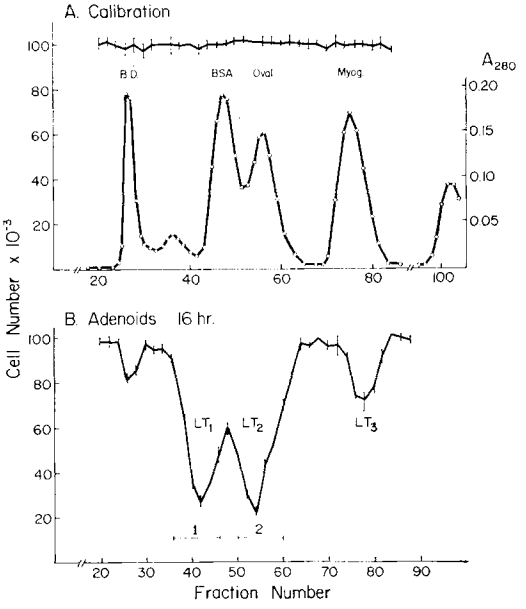

FIG. 1. Gel filtration chromatography of SAL collected after 16 hr from PHA-stimulated human adenoid lymphocytes *in vitro*. A. Column Calibration: Markers were suspended in a total volume of 2.0 ml PBS/SPF and eluted with reverse flow on a 2.5 × 100 cm Sephadex G-150 column. Markers employed were 6 mg each of bovine serum albumin (BSA), ovalbumin (Oval) and myoglobin (Myog) or cytochrome C (Cyt C) plus 0.1 ml of a blue dextran (B.D.) and phenol red (P.R.) solution. Also shown is a media control in which MEMS containing 20 μg/ml PHA-P and 3% FCS was concentrated 30× and 2.0 ml applied to the column. Column fractions were collected and 0.2 ml assayed for toxic activity. B. Chromatography of 2.0 ml of 25× concentrate from SAL taken after 16 hr of PHA stimulation. Column fractions were collected, sterile filtered and assayed in duplicate for LT activity by adding 0.2 ml to a 1 ml preestablished L-cell monolayer as described in Methods.

Kinetics of Appearance of the Various LTs in SAL Obtained from Cultures of PHA Stimulated Human Tonsil Lymphocytes

Lee and Lucas (14) reported that supernatants from PHA-stimulated human splenic lymphocytes contain two LT molecules, separable by sephadex gel filtration. These materials were identified as "early LT" and "late LT" based on the observation that the late LT was detected in SAL only after 36 hr in serum-free culture. On comparison, it appears that "late" and "early" LT components may correspond to our LT_1 and LT_2 activities, respectively, based on the similarity of their Sephadex G-150 elution profiles.

We decided to investigate the kinetics of appearance of the various LT activities in SAL obtained under our culture conditions. Cultures of human tonsil lymphocytes were established for either 1, 3 or 5 days in MEM containing PHA and 3% fetal calf serum as described in Materials and Methods. At the end of each incubation period, the cells were removed, and the supernatants collected and concentrated. The results of these experiments can be seen in the data presented in Fig. 2. Figure 2A shows the sephadex elution profile of 2.0 ml of 25× concentrated SAL collected 18 hr after PHA activation. Three peaks of LT activity are seen corresponding to the LT_1, LT_2 and LT_3 activities, respectively. We found that supernatants collected as early as 12 hr after PHA activation contain both LT_1 and LT_2. The relative amounts of LT_1 and LT_2 vary dramatically from culture to culture. However, LT_1 generally constitutes 30–60% of the total LT activity 24 hr after PHA stimulation. The amount of toxic activity associated with LT_3 is estimated to be around 10%.

The elution profile of SAL collected 3 days after PHA stimulation is shown in Fig. 2B. In this experiment, SAL were concentrated 10× and 2.0 ml applied to the column. Again, LT_1 and LT_2 activities are present. Their column distribution indicates that each activity is present in about 50%. However, the toxic activity associated with LT_3 is not apparent on day 3.

Since the LT activity in SAL collected 5 days after PHA stimulation is high, they were chromatographed without concentration. Figure 2C shows the sephadex elution profile of 2.0 ml of a 5 day SAL. Again, LT_1 and LT_2 are observed, while LT_3 is absent. Note that some toxic activity was detectable eluting in the void volume. This activity is present in about 70% of the experiments and is not unique to a 5 day SAL.

The results of these kinetic experiments indicate that all three LT activities are present within 12 hr after lymphocyte activation. However, while LT_1 and LT_2 persist in culture through day 5, LT_3 is not detectable after day 3.

Comparison of Multiple Lymphotoxin Molecules Secreted by Human Lymphocytes Activated with Various Mitogens

We next decided to determine whether different mitogens would cause secretion of different cytotoxic molecules. Lymphocyte cultures were established using human tonsils and the cells activated with optimal doses of either PHA (20 μg/ml) or concanavalin A (2 μg/ml) in MEM containing 3% FCS. After a 24 hr incubation, the SAL from each culture were collected, concentrated, and 2.0 ml samples chromatographed as in previous experiments. The SAL obtained from PHA or Con A-stimulated cells were concentrated 25× or 40×, respectively, before chro-

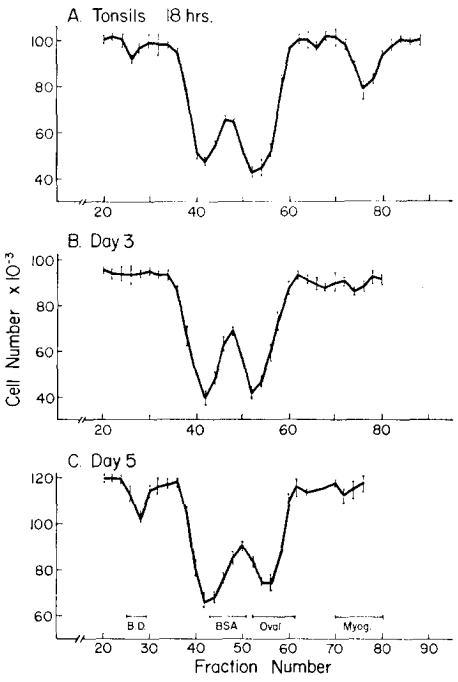

FIG. 2. Sephadex chromatography of SAL obtained from PHA-stimulated human tonsil lymphocytes after 1, 3, and 5 days in culture. A. SAL were obtained after 18 hr of PHA stimulation, concentrated 25×, and 2.0 ml applied to the column. Column fractions were collected, filter sterilized, and 0.2 ml assayed for LT activity. Vertical bars represent error in cell counts from duplicate tubes. B. Same as A, except SAL were collected after 3 days of stimulation and 2.0 ml of a 10× concentrated sample was chromatographed. Fractions were collected, and 0.2 ml assayed for LT activity. C. Same as A, except SAL were collected after 5 days of stimulation, and 2.0 ml of unconcentrated sample was chromatographed. Fractions were collected, and 0.2 ml assayed for LT activity.

matography. The results shown in Figs. 2A and 3 indicate that within 24 hr, both mitogens will induce human tonsil lymphocytes to secrete LT_1 and LT_2. LT_3, however, was not detected in SAL produced by Con-A stimulation.

TABLE 1

Additivity of Pooled Fractions of LT_1 and LT_2 When Assayed on Alpha L-929 Target Cells

LT Fractions	Activity (Unit/ml)
100% LT_1	63 ± 2
100% LT_2	31 ± 2
50/50 LT_1/LT_2	44 ± 3

Pooled fractions of LT_1 and LT_2 were obtained from the column shown in Fig. 1B. Pool 1 (LT_1) was concentrated 5× from an initial volume of 45 ml and Pool 2 (LT_2) was concentrated 5× from an initial volume of 60 ml, using an Amicon Ultrafilter and a PM10 membrane. Toxicity was assayed as described in Methods.

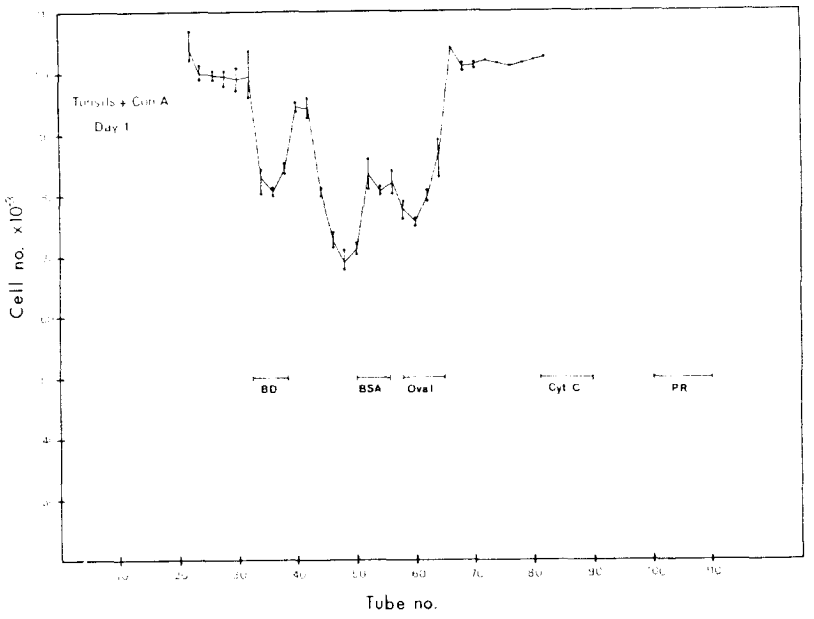

FIG. 3. Chromatography of SAL from 1 day Con A stimulated cultures of human tonsil lymphocytes. Cultures of human tonsil lymphocytes were established as in Materials and Methods. After 24 hr incubation, the SAL were collected, concentrated 40× and 1.5 ml applied to the sephadex column. Fractions were collected, filter sterilized, and 0.2 ml tested for toxic activity.

Additivity of Toxic Activity from Pools of LT_1 and LT_2 when Tested on L-Cell Targets In Vitro

To determine whether toxic activity in fractions of LT_1 and LT_2 was additive or synergistic when assayed together on target L-cells, pooled fractions of LT_1 and LT_2 were obtained. Pool 1 (LT_1) and Pool 2 (LT_2) were collected from the column shown in Fig. 1B and each concentrated 5× by ultrafiltration. Toxicity was measured as described in Methods, employing samples containing 100% LT_1, 100% LT_2, or a 50/50 mixture of each component. The results shown in Table 1 indicate that LT_1 and LT_2 act in an additive manner when assayed together on L-cell targets.

Detection of Multiple LT Molecules within Activated and Nonactivated Human Tonsil Lymphoid Cells

Experiments were initiated to determine whether intracellular cytotoxic activity could be detected within mitogen-activated lymphocytes. Lymphoid cells obtained from human tonsils were employed throughout these studies. Cells were obtained and established for 1, 3, or 5 days in 150 ml cultures in MEMS plus 20 μg/ml PHA at a density of 4×10^6 cells/ml. As a day 0 control, cells were washed and disrupted as described in Materials and Methods. After 1, 3, or 5 days of incubation, the cells established in culture were collected by sedimentation, the whole cell pellet washed thoroughly with PBS, resuspended in fresh MEMS, and disrupted. After sedimentation of the cellular debris, the supernatant was filter sterilized, and tested for toxic activity. At the same time, the corresponding culture supernatants

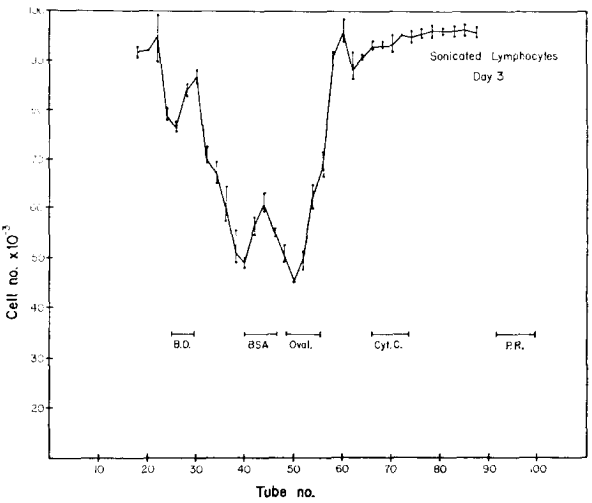

FIG. 4. Chromatography of homogenates from 3-day cultures of PHA-activated tonsil lymphocytes. Tonsil lymphocytes (1.5×10^6) were established in culture at 4×10^6 cells/ml and stimulated with PHA-P. After 72 hr at 37°C, the cells were collected, washed with 150 ml PBS, resuspended in 10 ml MEM, and sonicated as described in Materials and Methods. The homogenate was clarified by centrifugation at 15,000*g*, 15 min and the supernatant collected and concentrated 3-fold. 2.0 ml was applied to the sephadex column and fractions were collected and 0.2 ml assayed for LT activity.

were also collected, filter sterilized, and tested. The results of these experiments are shown in Table 2. Unstimulated (day 0) cells have very low levels of intracellular toxic activity, however, within 24 hr after stimulation, the levels in an identical number of cells have increased 400-fold. The intracellular toxic activity increases another 10-fold up to day 3 and by day 5, decreases below the levels seen on day 1. However, the total toxic activity in the supernatant continues to rise to a very high level on day 5. Experiments were also performed to be sure that the observed intracellular toxic activity was not due to contamination by cell bound activity. Before the cells were disrupted, they were washed extensively (using up to 300 ml PBS) and the total cytotoxic activity in the last washing was tested. These results (Table 2) show that total intracellular toxic activity ranges from 6 to 30 times higher than the activity in the last washing.

We also designed experiments in attempts to demonstrate that the observed intracellular cytotoxic activity was not due to nonspecific cytolytic material(s) unrelated to LT, *i.e.*, lysosomal enzymes. We homogenized similar numbers of alpha L-cells and HeLa cells with the cell numbers adjusted to equivalent cell volume rather than cell number, considering the diameters of these cells to be 20 and 15 microns, respectively. In no case was nonspecific toxicity evident when L-cell or HeLa cell homogenates were assayed on L-cell monolayers (Table 3).

In addition, previous studies revealed that human LT-induced cytolysis of L-cells *in vitro* has a very sharp temperature optimum, *i.e.*, 95% inhibited by a shift from 37°–34°C (18). We tested the activity of intracellular cytotoxicity from the day 0 unstimulated lymphocyte homogenates at both the permissive (37°C) and nonpermissive (34°C) temperatures and found it was virtually totally inhibited at 34°C (Table 3). The intracellular toxicity of 1- and 3-day PHA-stimulated

lymphocyte homogenates is also totally blocked at the nonpermissive (34°C) temperature (data not shown). Additional studies to be published elsewhere revealed that rabbit anti-human LT blocked a large portion of the intracellular LT activity. In addition, previous experiments revealed that the cell disruption processes themselves were not destructive to the LT molecules.

Sephadex Chromatography of Intracellular Human LT Activity Obtained from 3-Day Cultures of PHA-Stimulated Lymphoid Cells

A 10 ml cell homogenate was prepared from 1.5×10^9 lymphocytes 3 days after culture with PHA as described in Materials and Methods. The homogenate was concentrated to 2 ml by ultrafiltration through a PM10 membrane filter. Sephadex G-150 gel filtration chromatography of this sample revealed at least two peaks of toxic activity corresponding to the elution profiles of LT_1 and LT_2 (Fig. 4). There is also a small amount of toxic activity eluting in the void volume. However, there is no noticeable activity in the 10–20,000 MW range.

DISCUSSION

Lymphotoxin activity, measurable in supernatants from a stimulated human lymphoid cells *in vitro*, appears to be due to a multi-component family of cytotoxic materials. Bolous *et al.*, employing gel electrophoresis, detected two LT activities identified as having a slow and a fast mobility. They also observed what appeared to be "LT aggregates" during sucrose gradient centrifugation (12). Jeffes and Granger (19) and Peter *et al.*, (13) reported two separable peaks of LT activity when human SAL were fractionated on DEAE-cellulose. The former investigators reported that while both have the same molecular weight, the first peak elutes just behind human gamma globulin, and the second elutes with albumin (19). In another study, Walker and Lucas identified two separable LT-like activities with

TABLE 2

Total LT Activity in Supernatants, Washings, and Cellular Homogenates of Activated and Non-activated Human Tonsil Lymphocytes

	Culture supernatant	Last washing	Intracellular LT	Intracellular LT/10^8 cells	Ratio of extracellular LT/intracellular LT
Day 0	—	—	<2	<0.33	—
Day 1	6,000 ± 240	90 ± 2	825 ± 40	138	7.0
Day 3	18,000 ± 1,350	180 ± 11	6,125 ± 450	1,020	3.0
Day 5	41,625 ± 550	31 ± 3	180 ± 3	30	130

Lymphocytes were obtained and a) established in 150 ml cultures in MEMS plus 20 μg/ml PHA at a density of 4×10^6 cells/ml for either 1, 3, or 5 days or b) washed with 30 ml PBS (day 0) and disrupted as described in Methods. The cells established in cultures were collected by sedimentation, washed with a total of 300 ml PBS/1% NBCS, finally resuspended in 7.5 ml MEMS and sonicated. After removal of the cellular debris, the homogenate was sterile filtered and tested for toxic activity as described in Methods. LT activity is expressed as the total amount in the entire supernatant or homogenate. Lymphocyte viability was determined by microscopic observation of cells suspended in 0.1% Eosin Y, and was high during days 1 to 3, but dropped to 60–70% by day 5.

TABLE 3

Toxicity of Unstimulated Lymphocyte and Non-lymphoid Cell Homogenates on Alpha-L Cell Targets[a]

Cell source for homogenate	Homogenate dilution	Target L-cell viability when incubated at 37°C	34°C
Unstimulated[b] lymphocytes (3×10^9)	1/2 (Eq of 3×10^8 cells)	77	96
	1/5	89	97
	1/25	99	99
Alpha-L cells[c] (1.2×10^8)	Undiluted (Eq of 13×10^6 cells)	120	—
	1/4	107	—
	1/16	97	—
HeLa Cells[d] (3×10^8)	Undiluted (Eq of 2×10^7 cells)	108	—
	1/4	102	—
	1/16	98	—

[a] Each LT assay was performed as described in Materials and Methods and results are expressed as the average of triplicate points for each dilution.

[b] Tonsil lymphocytes (3×10^9 cells) were collected and washed with 20 ml PBS/1% NBCS, resuspended in 10 ml MEM and disrupted as described in Materials and Methods. The homogenate was centrifuged, filter sterilized and various dilutions tested for toxic activity at both 37°C and 34°C on 10^5 alpha-L cell targets.

[c] Alpha-L-929 mouse fibroblasts (1.2×10^8 cells) were obtained by trypsinization of stock monolayer cultures. The cells were washed with 20 ml PBS and finally resuspended in 9.0 ml MEM and sonicated. The homogenate was treated and tested for toxicity as described for *b*.

[d] HeLa cells (3×10^8 cells) were obtained in an identical manner as alpha-L cells. After washing with PBS, the cells were suspended in 15 ml MEM and disrupted. The homogenate was treated and tested for toxic activity as described for *b*.

different molecular weights of 76,000 and 45,000 daltons by chromatography on Sephadex G-100 (20). The present studies demonstrate the existence of three LT components separable on the basis of size, termed LT_1, LT_2, and LT_3, with molecular weights of 80–90,000; 50,000; and 10–15,000 daltons, respectively. Furthermore, these represent families of molecules, and subsequent publications will demonstrate that both LT_1 and LT_2 can be further separated into multiple components. It is interesting to note here that certain continuous human lymphoid cell lines (CLCL) spontaneously secrete LT molecules (10). Recent studies in our laboratory suggest that the CLCL PGLC-33H, RPMI-1788 and Moore's 5287 all appear to only secrete members of the LT_1 family of cytotoxins.

The relationship of the different families, LT_1, LT_2, and LT_3, to each other is not yet clear. However, immunologic and physical-chemical data strongly suggest that the LT_1 and LT_2 families are not related. The relationship of the human LTs to the LTs studied in other animal species (mouse, rat, and guinea pig) is also not clear. However, they also appear to be composed of populations of physically different materials (21–25). There is very little information on the physical-chemical nature of LT_3. Material which elutes in the void volume, greater than 150,000 MW, appears sporadically and may only be aggregates of the LT_1 family. However, additional experiments are under way to verify this concept.

There are important technical considerations which could explain why different members of the LT families are detected in various laboratories. The first reason is that each individual component is present in a given supernatant at a different concentration. Therefore, it is imperative to start with a sufficient amount of each component, so that it is still detectable after fractionation. Also of major importance is the type and sensitivity of target cell employed in the cytotoxic assay. In addition, the proper amount of each individual fraction must be tested in order to effectively resolve closely related materials or detect minor components. For example, at higher concentrations, LT_1 and LT_2 fractions from chromatography on Sephadex G-150 run together to form one broad peak of toxicity, yet when smaller amounts are measured, two distinct peaks of activity are resolved. Finally, the investigator must be aware of sequential appearance and different stabilities of each of the individual members.

The actual amount of each of the different LT componetns in a given supernatant varies considerably and they are difficult to assess quantitatively. There was, however, no unique early or late molecules associated with the LT_1 and LT_2 families measurable in these studies, as previously reported (14). However, LT_3 activity was not detectable after 3 days in these cultures. Recent evidence indicates the LT_2 family is highly unstable, and the amounts detected in a supernatant may only represent a fraction of the actual amount secreted by the activated cell. Measurements by several methods indicate LT_1 may be from 30–90%, LT_2 from 10–70%, and LT_3 no more than 10% of the total activity in a given supernatant. Also, cells from different human lymphoid tissues activated by either Con-A or PHA are able to secrete similar cytotoxins *in vitro*. While cells obtained from tonsils, adenoids and peripheral blood are able to secrete LT_1 and LT_2, only adenoids and tonsils were able to release detectable amounts of LT_3. However, this situation is obviously complex, and the secretion of these various cytotoxins must be governed by strict regulatory control mechanisms.

Lymphotoxin-like molecules were also detectable within the activated lymphoid cells themselves. This cytolytic activity is believed to be due to LT for the following reasons: a) analogous amounts of extract from nonlymphoid cells had no cytolytic activity; b) the cytolytic activity was suppressible by the restrictive temperature of 34°C, when tested on L-cells; c) the cytotoxic materials have the same basic molecular size as LT_1 and LT_2; and d) antibodies specific for the human LT cytotoxins partially inhibit the cytotoxic reactions caused by the unseparated homogenate. While unstimulated human lymphoid cells contain low levels of intracellular LT, after activation these levels increase dramatically up to day 3, and thereafter drop dramatically.

There appears to be a regulatory control mechanism(s) operative on the synthesis and secretion of the LT_1 family of effector molecules during mitogen stimulation of human lymphocytes *in vitro*. In the present studies, we found that intracellular levels of LT increase dramatically up to day 3, and then decline rapidly by day 5. In contrast, extracellular levels of LT increase rapidly when the intracellular levels are high and plateau when the intracellular levels decrease. These studies suggest a possible control mechanism(s) which terminates actual LT biosynthesis within the activated cell. These studies plus previous reports indicate that the amount of LT in the extracellular *milieu* is controlled by at least two separate mechanisms. The first deals directly with the secretion process itself, a

secretory system which is capable of being activated or inactivated very rapidly by membrane contact with the stimulating agent (*26–27*), and second, dealing with the actual biosynthesis of LT within the cell, which may be affected by a feedback system.

The present studies indicate that a lymphocyte is capable of secreting a group of molecules with cytotoxic or cytostatic activities, which it could employ as effectors in tissue destructive CMI reactions. It is possible that different members of the LT family may be effectors in different cytolytic reactions depending upon the nature of the activating agent or the type of target cell and tissue involved.

ACKNOWLEDGMENTS

The authors are grateful to St. Josephs Hospital, Orange, California, and Placentia-Linda Hospital, Yorba Linda, California, for providing tissue. We also thank Gloria Stangl for preparation of this manuscript and Robert Yamamoto for excellent technical assistance.

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