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Journal

Molecular Immunology, 16(3)

ISSN

0161-5890

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Publication Date

1979-03-01

DOI

10.1016/0161-5890(79)90144-5

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RELEASE OF LT MOLECULES WITH RESTRICTED PHYSICAL HETEROGENEITY BY A CONTINUOUS HUMAN LYMPHOID CELL LINE *IN VITRO**

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(Received 13 March 1978)

Abstract—Lymphotoxins released by lectin activated human lymphoid cells *in vitro* are complex and can be resolved by molecular sieving techniques into multiple classes of activity, termed complex, α , β and γ . The classes can be further resolved into sub-classes on the basis of their charge by ion-exchange chromatography and electrophoresis. We found the lytic activity spontaneously released by the continuous human B lymphoid cell line, PGLC-33H, to be of limited physical heterogeneity, and essentially identical to the α mol. wt class of human LT⁺ molecules. This was determined by functional, physical-chemical and immunologic means. We found that most of the α -LT charge sub-classes present in supernatants from mitogen stimulated normal lymphocyte cultures were also detected in these supernatants. These studies suggest: (a) this cell line provides a source of human LT for study with restricted heterogeneity; (b) different lymphoid cells (T and B) may be capable of releasing different LT molecules; and (c) use of selected human lymphoid cell lines may be useful to examine the biokinetics of LT synthesis and release.

INTRODUCTION

In-vitro stimulation of lymphocytes from experimental animals and man by specific (antigens) or non-specific (mitogens) means results in the release into the culture medium of a complex family of soluble molecules (Lawrence & Landy, 1969; Granger, 1972; Ruddle, 1972). It has been suggested that these materials, termed lymphokines (LK), may be important effectors in causing the various manifestations of cell-mediated immunity (CMI) observed *in vitro* and *in vivo*. One family of LK, termed lymphotoxins (LT), has been shown to be cytotoxic or growth inhibitory to cells *in vitro*, depending on the concentration of the cytotoxin and the particular indicator target cell employed (Williams & Granger, 1973; Jeffes & Granger, 1976; Walker & Lucas, 1972; Namba & Waksman, 1975). The actual role of LT in CMI reaction is not yet clear. However, it is becoming more plausible, for several studies, employing antisera which will neutralize their activity *in vitro*, indicate they are lytic effectors in certain types of lymphocyte induced target cell destructive reactions *in vitro* (Gately *et al.*, 1976; Hiserodt & Granger, 1977).

The human LT family of effectors is complex and can be separated into various classes based on

differences in their mol. wt (Walker *et al.*, 1976; Granger *et al.*, 1978) and several classes can be further resolved into sub-classes on the basis of differences in their charge (Lee and Lucas, 1976; Hiserodt *et al.*, 1976a; Granger *et al.*, 1978). The first class, complex, is greater than 150,000 daltons, the second, α , is 70–90,000 daltons. Both classes are relatively stable to heating, storage and manipulation (Walker *et al.*, 1976b; Granger *et al.*, 1978). The α class contains 3–6 sub-classes, which can be resolved by chromatography on diethylamino ethyl cellulose and phosphocellulose (Lee & Lucas, 1976; Hiserodt *et al.*, 1976b, Granger *et al.*, 1978). The third class, β , is composed of at least two sub-classes; one relatively stable, the other highly unstable (Hiserodt & Granger, 1976a). Finally, a third, short-lived small mol. wt class, termed γ , of 10–15,000 daltons, which is highly unstable and little studied, has also been described (Hiserodt *et al.*, 1976b).

In order to obtain large quantities of human LK for purification from both a uniform source and identifiable cell type, a number of investigators have examined supernatants from continuous human lymphoid cell lines. These studies revealed that many human lymphoid cell lines are spontaneously releasing LK-like materials (Granger *et al.*, 1976; Papageorgiou *et al.*, 1972; Amino *et al.*, 1974). Several studies reported LT activity in the supernatants from different cell lines possessed physical-chemical properties similar to those described for the α -LT class (Granger *et al.*, 1976; Amino *et al.*, 1974). However, the activity detected in these supernatants was only partially characterized. We have shown *in vitro* that lectin activated human T and B cells can release LT, and moreover, T cells can express LT on their surface plasma membranes (Hiserodt *et al.*, 1977). It has been shown that the human continuous B cell line PGLC-

*This work was supported by the Carcinogenesis Training Grant, National Institutes of Health, No. 09054, the Rheumatic Diseases Research Foundation, No. 1882 and the Institute of Allergy and Infectious Diseases, NIH, AI-09460.

† Abbreviations used: LT, lymphotoxin; SAL, supernatant from cultures of mitogen activated human lymphoid cells; MIF, migration inhibition factor; MC, mitomycin C; PBS, phosphate buffered saline; FCS, fetal calf serum; NCS, newborn calf serum; MEM, minimal essential medium; PHA, Phytohemagglutinin.

33H releases MIF and LT-like material(s) (Granger *et al.*, 1970; Papageorgiou *et al.*, 1972). We have more carefully examined the physical-chemical properties of LT-like activities present in the supernatant of a continuous human lymphoid cell line of B cell origin, PGLC-33H. We found that this cell line only released LT molecules with properties of the α mol. wt LT class. Additional study revealed all but one of the α -LT charge sub-classes released by mitogen activated human lymphocytes detectable in ion-exchange columns is present.

MATERIALS AND METHODS

Tissue culture reagents and cell lines

The mouse α -L-929 cell line was employed as an LT target cell in these *in-vitro* studies (Spofford *et al.*, 1974). Cells were maintained in 3% newborn calf serum (NCS) or fetal calf serum (FCS) in Eagle's Minimal Essential Medium (MEM), supplemented with 0.29 mg/ml glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin. Cells were passed at bi-weekly intervals.

Both the PGLC-33H continuous lymphoid cell line (generously supplied by Dr. P. Glade, Pediatrics Department, University of Florida, Gainesville, FL) and the HeLa cell line were maintained in 10% FCS in MEM as described above. However, PGLC-33H cultures were also supplemented with MEM levels of sodium pyruvate and non-essential amino acids.

Preparation and concentration of supernatants from activated lymphocytes (SAL)

Human tonsil and adenoid lymphocytes were prepared as a single cell suspension as described previously (Kramer & Granger, 1975). The lymphocytes in these suspensions were adjusted to 5×10^6 viable cells/ml in MEM containing 10% NCS, non-essential amino acids and 1 mM pyruvate. Phytohemagglutinin-P (PHA) (Difco, Los Angeles, CA) was then added to a final concentration of 20 μ g/ml and the cultures incubated in 32-oz prescription bottles for 72 hr at 37°C in a 5% CO₂, 95% air atmosphere. The medium was cleared of cells by centrifugation at 300 g for 10 min, and used immediately. PGLC supernatants were collected after 2–3 days of growth at 37°C, in MEM + 10% FCS. The medium was cleared of cells by centrifugation (300 g for 10 min) and either stored at –20°C or used immediately. Supernatants from both tonsils and PGLC lymphocyte cultures were routinely concentrated 3–20-fold by ultrafiltration utilizing an Amicon PM-30 and/or a PM-10 Diaflo Membrane. Extensive tests revealed all of the LT activities were retained in the concentrate, and no activity was detectable in the filtrate.

Lymphotoxin assays

Inhibitory or LT assays were performed, employing: (1) growing HeLa; (2) growing α -L-929; or (3) mitomycin C (MC) treated nondividing α -L-929 cells. The details of these assays have been reported elsewhere (Jeffes & Granger, 1976; Spofford *et al.*, 1974). Briefly, target cells were established as monolayers in 1 ml tube cultures containing 100,000 cells during an overnight pre-incubation at 37°C in MEM. To determine the amount of LT present, spent media was discarded and serial dilutions of LT in fresh media were added to each monolayer of target cells. These tube cultures were incubated at 37°C for an additional 24–110 hr (in the case of growing HeLa cells). After this incubation, the number of viable adherent cells was determined on a Model F Coulter Counter (Kramer & Granger, 1976). One unit of LT activity is defined as that amount which will destroy 50,000 L cells. The reciprocal of the lowest dilution that results in a 50 reduction ($1:1_{50}$) in the target L cell number of units of activity/ml in the original sample supernatant.

Sephadex chromatography

A Sephadex G-150 (Pharmacia, Uppsala, Sweden) column (2.5 × 90 cm) was equilibrated in 20 mM potassium phosphate, pH 7.6, and was calibrated with Blue Dextran (2 × 10⁶ daltons), bovine serum albumin (68,000), ovalbumin (44,000) and phenol red (354) applied in a total volume of 2.0 ml. Five millilitre fractions were collected and monitored for absorbance at 280 nm. Two millilitres of SAL, PGLC or control supernatants were chromatographed over this column, and 0.05–0.2 ml samples of the eluted fractions were added directly to 1 ml cultures of MC-treated L-929 target cells. The amount of destruction was determined after 16 hr incubation at 37°C. When growing target cells were employed, every other fraction from the sephadex column was tested. This method of assessing LT activity was employed in all column chromatography experiments.

Diethylamino ethyl-cellulose (DEAE-cellulose)

A 2.5 × 34-cm column of Whatman DE-11 resin was equilibrated in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA. Protein was eluted with a linear 500 ml gradient from 0 to 0.3 M NaCl, followed by 50 ml of a 1.0 NaCl step in the same buffer. Six ml fractions were collected and the conductivity and absorbance at 280 nm were monitored.

Phosphocellulose (PC)

A 2.5 × 10-cm column of Whatman PC was equilibrated in 10 mM potassium phosphate, 0.1 mM EDTA (pH 6.6). Samples were eluted with a 500 ml linear gradient from 0 to 0.3 M NaCl. Six ml fractions were collected, and the conductivity and absorbance at 280 nm were monitored.

Polyacrylamide disc gel electrophoresis (PAGE)

A 0.1 ml sample in 20% sucrose was applied to a 0.5 × 8.0 cm separating gel (7% acrylamide) with a 1 cm stacking gel, according to the method of Davis (1964). The samples were electrophoresed at 4 mA/gel at 4°C, and were then cut into 2 mm slices. Lymphotoxin activity was eluted from each slice by incubation in 0.3 ml of MEM and 3% FCS or PBS for 18 hr at 4°C, and then 0.1 ml was tested for toxic activity on MC treated L cells in tube culture.

RNase assay

The assay for RNase activity was carried out under the following reaction conditions: To 0.02 ml of a ³H or ¹⁴C-labelled purified mixture from yeast and drosophila (kindly provided by Drs. C. McLaughlin and J. Manning, Department of Molecular Biology and Biochemistry, University of California, Irvine, CA) was added 0.1 ml of 10X SSC, 0.3 ml of 10 mM MgCl₂, 10 mM CaCl₂, and 0.1 ml of the LT containing test or control sample to be evaluated. Following an incubation for 30 min at 37°C, 0.1 ml of bovine serum albumin (2 mg/ml) as carrier, and 0.6 ml of cold 10% trichloroacetic acid were added.

The samples were permitted to stand overnight at 4°C, and the precipitates collected on Whatman GF/C filters. Radioactivity in these precipitates was monitored by using 6.0 ml of Omnifluor liquid scintillation cocktail and counting for 10 min in a Beckman LS-233 liquid scintillation counter.

Neutralization of LT activity

Samples containing human LT activity were incubated in rabbit anti- α LT class antisera (from A series animals) which will neutralize *in vitro* all members of the α class, as previously described (Lewis *et al.*, 1977; Yamamoto *et al.*, 1978). Briefly, animals were immunized with fractions obtained from molecular sieving columns containing all the human α mol. wt LT class molecules. These preparations were derived from 3 to 5 day supernatants obtained from PHA stimulated normal human lymphocytes and were free of all other mol. wt LT classes. This particular lot of antiserum, when tested against other mol. wt LT classes was a potent inhibitor for α and complex class LT molecules but was not reactive with

Table 1. The capacity of lytic activity released by the cell line PGLC-33H to bind to target L cells *in vitro* and to induce cytolysis at different temperatures

Experiment	Exposure to PGLC-LT		
	1 hr at 37°C ^a	24 hr at 37°C	24 hr at 34°C
1	3 ± 1 ^b	220 ± 10	8 ± 1
2	8 ± 1	61 ± 8	13 ± 3

^a Tube cultures of target monolayer L cells were exposed to serial dilutions of LT-containing medium. After 1 hr, this media was discarded, and the monolayer washed and replaced with fresh media. Cultures were assayed for the units of detectable LT activity after 20 hr, as described in the text.

^b Activity is expressed as units of LT/ml supernatant, determined as described in the text.

members of the β or γ classes. After overnight incubation at 4°C, rabbit antisera LT complex was precipitated by addition of either goat anti-rabbit IgG or sheep anti-rabbit serum. After an additional 12 hr of incubation at 4°C, the precipitate was removed by centrifugation, and the supernatant was diluted with fresh media containing 3% NCS. Duplicate samples of each dilution were tested on MC treated L-929 cells.

RESULTS

Assessment of binding, growth inhibition and effect of temperature on lysis of target L cells in vitro by PGLC-33H supernatants

The mechanism of *in vitro* target L-cell destruction induced by LT generated from mitogen activated human lymphocytes at least two parameters. First, LT may rapidly bind to the target cell (Hessinger *et al.*, 1973) and second, the cytolytic step of the reaction appears to be temperature dependent. Kramer and Granger (1976) reported a 97% reduction in LT

mediated cytodestruction, if the lytic reactions were incubated at 34°C, instead of 37°C. We decided to examine if LT released by PGLC-33H cells behaved in a similar fashion.

Serial dilutions of PGLC or control supernatants (concentrated 10-fold) were made in MEM + 3% FCS, (MEMS) and added to three parallel sets of duplicate tubes containing mitomycin C treated L cells. Two sets of tubes were then directly incubated at either 37°C or at 34°C, without further manipulation. The remaining set of tubes were incubated for 1 hr at 37°C, and the free LT removed by washing the monolayers 5 times with a total of 15 ml of PBS. After the addition of fresh MEMS, these tubes were incubated for 20 hr at 37°C, the total adherent cell number in all cultures was determined. The number of LT units detected in each set of tubes is shown in Table 1. First, a small but significant amount of PGLC-LT did bind to the L-929 target cells after a 1 hr incubation at 37°C. Second, the activity of PGLC-LT was dramatically reduced by incubating the cultures at 34°C, when compared with the cultures incubated at 37°C.

It has been previously suggested that LT released by activated human lymphocytes at low, sub-toxic levels can cause growth inhibition of cells *in vitro* (Jeffes & Granger, 1976; Walker & Lucas, 1972; Namba & Waksman, 1975). In order to continue a functional comparison of the LT released from mitogen activated lymphoid cells with the LT-like material secreted by PGLC-33H cells, we tested the ability of the latter cytotoxin(s) to growth inhibit HeLa target cells, as previously described (Jeffes *et al.*, 1976). PGLC or control supernatants were concentrated 10-fold, serially diluted, and cultured on growing HeLa cell monolayers for 48 hr at 37°C. In addition, the same concentrates were tested on MC treated L-929 cells to determine the number of units of cytolytic activity in

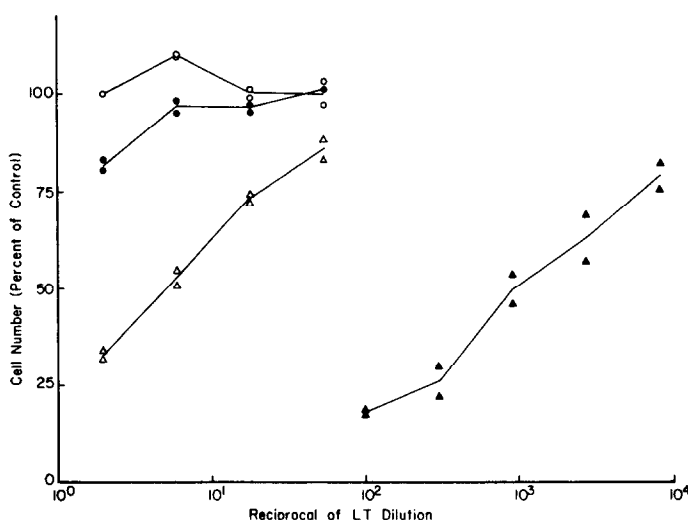


Fig. 1. The effects of PGLC-33H supernatants on growth inhibition of HeLa cells and on cytolysis of mitomycin C treated L-929 cells. All supernatants were concentrated 10-fold and subsequently serially diluted. The numbers of HeLa cells in the presence of PGLC-33H supernatants (Δ), concentrated media (\circ), and L-929 conditioned media (\bullet) are expressed as per cent of control HeLa cell cultures which received only fresh media and no concentrates after 48 hr. The numbers of remaining L-929 cells (\blacktriangle), after 48 hr in the presence of PGLC-33H supernatants are expressed as the per cent of control mitomycin C treated cells which received only fresh media.

Table 2. Heat stability of PGLC-LT cytotoxic activity measured *in vitro* on mitomycin-C treated L-929 cells

Experiment	Temperature ^a			
	37°C	56°C	70°C	85°C
1	150 ± 30	130 ± 20	75 ± 15	10
2	N.D. ^b	320 ± 50	250 ± 50	10

^aIncubation of LT containing SAL was carried out for 15 min at each temperature. Then serial dilutions of each sample were assessed on L cells and the unit of LT activity remaining was determined as described in the text.

^bNot determined.

each preparation. Figure 1 illustrates the results of these experiments. It can be seen that a dilution of about 1:5 causes 50% decrease in the expected number of HeLa cells after 48 hr. No effect was observed for control supernatants of concentrated MEM-S or L-929 conditioned medium. These same supernatants contained a high level of LT activity (950 units/ml) when measured on MC treated L-929 targets. PGLC supernatants were capable of promoting a concentration dependent cytolysis or growth inhibitory effect. Furthermore, the cytolytic effect manifested on L-929 cells was approx 100–200 times more sensitive than that effect observed on HeLa cells. This same result had also been shown for supernatants from mitogen activated lymphocytes (Jeffes & Granger, 1976).

Several investigators have reported a cell-lytic activity detected *in vitro* in supernatants from different continuous human lymphoid cell lines possessed physical characteristics similar to the α class of lymphotoxins. We decided to examine the physical characteristics of the lytic activity released by PGLC-33H cells in light of new data on multiple LT classes

and sub-classes. Particularly important was to examine the possibility that a continuous lymphoid cell line may only secrete a single LT class or sub-class in a fashion analogous to the production of a single Ig product seen with both human and murine myeloma cells.

Heat stability of PGLC-LT

We first performed a heat inactivation study; the results of which can be seen in Table 2. Supernatants were heated and dilutions tested for LT activity, as described in Materials and Methods. As previous investigators have shown for supernatants from both continuous culture lymphoid cells (Amino *et al.*, 1974) and mitogen activated human lymphocytes (Walker *et al.*, 1976; Peter *et al.*, 1973; Kolb & Granger, 1968), the lytic activity was destroyed at 85°C and stable at 37°C and 56°C, following 15 min exposure at each given temperature.

Gel filtration

In order to determine the number of LT classes, both the supernatants from PGLC-33H and PHA-P, activated human tonsil lymphocytes were fractionated by gel filtration. We did not store these supernatants, but they were fractionated immediately, in order to insure detection of the unstable β -class LT activity, if present. Supernatants were harvested, concentrated 10–17-fold, and then fractionated over Sephadex G-150. The data shown in Figs. 2(A) and (B) demonstrates that α class LT was the major LT class present in the PGLC supernatants. The lytic activity in PGLC supernatant eluted from this column with an apparent mol. wt of 70–90,000 daltons [Fig. 2(B)], whereas, the LT from mitogen activated lymphocytes eluted in two major classes α -LT at 70–90,000, and β -LT at 35–50,000 daltons [Fig. 2(A)].

In addition, the activity of PGLC-LT eluting from the Sephadex G-150 column was further tested on growing L-929 cells. Although the data are not shown, the ability of PGLC LT eluting from the column to growth inhibit L-929 coincided with the cytotoxic activity observed on MC treated L-929 cells [Fig. 2(B)]. In contrast, if dividing HeLa cells were utilized, inhibitory activity eluted from the sephadex column over a more broad range of apparent mol. wt (from 40 to 90,000 daltons). Next, PGLC-LT fractions and whole supernatants were tested for activity after three months of storage at 4°C, in order to monitor the stability of this material. The levels of activity and elution profiles of cytotoxic activity coincided with those observed upon initial fractionation. Hence, it

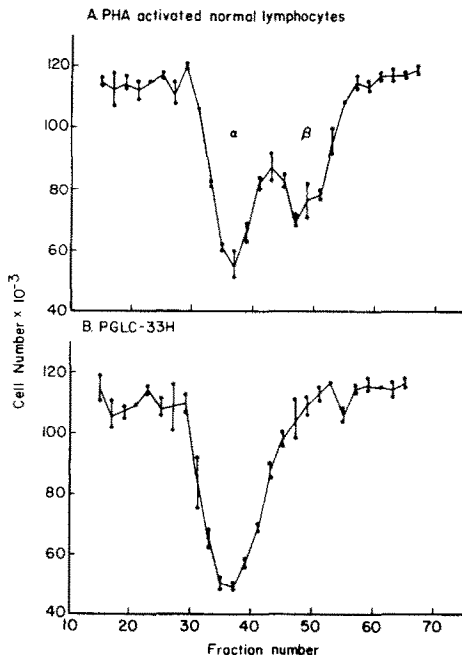


Fig. 2. Sephadex G-150 profiles of lymphotoxin activity released by PHA activated human tonsil lymphoid cells (top) and from the continuous lymphoid cell culture, PGLC-33H. Void volume is at fraction 19 and BSA elutes at fraction 42.

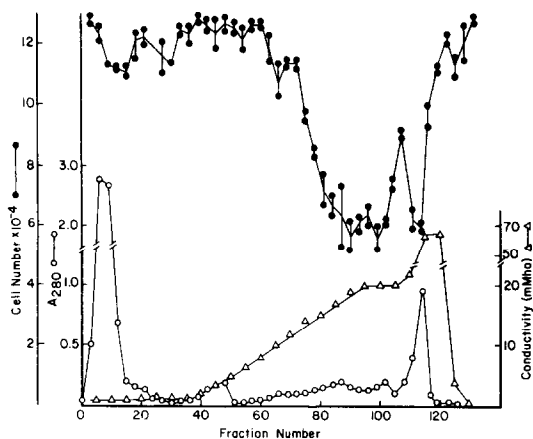


Fig. 3. Elution profile of DNase treated PGLC-33H supernatant off of phosphocellulose. The column is equilibrated in 10 mM potassium phosphate, 0.1 mM EDTA (pH 6.6) and the protein was eluted with a 0–0.3 M linear gradient of NaCl. Absorbance at 280 nm (\circ), conductivity at room temperature (\triangle) and lymphotoxin activity (\bullet) are plotted vs fraction number.

appears that PGLC released cytoxin(s) are composed of a stable, single molecular weight class similar to α -LT from mitogen stimulated human cells, which exhibits both cytotoxic and growth inhibitory activities on L-929 cells.

Ion exchange

Recently, Lee and Lucas (1976) demonstrated that the human α -LT class contains various sub-classes which can be separated on the basis of differences in their charge. To examine if the PGLC released cell toxin(s) contained sub-classes, we separated PGLC supernatants on phosphocellulose and DEAE-cellulose columns.

Initially, PGLC supernatants were concentrated 10-fold, dialyzed against PBS, and subjected to DNASE treatment (80 units pancreatic DNase A/ml) for 2 hr at room temperature in the presence of 10 mM $MgCl_2$, 10 mM $CaCl_2$. After dialysis in 10 mM potassium phosphate, 0.1 mM EDTA (pH 6.6), the PGLC-LT was chromatographed over phosphocellulose. As shown in Fig. 3, nearly all of the cytotoxic material was retained by this resin under these conditions. In several experiments, we found a small amount of activity did not bind to the column, and eluted with the first protein peak. The majority of lytic activity began to elute from the column after a concentration of 0.15 M NaCl, and a second peak was included with the 1 M NaCl wash.

The entire LT-containing eluate from the PC column was then concentrated to 5–10 ml by ultrafiltration and dialyzed against 50 mM Tris, 0.1 mM EDTA (pH 9.0) and applied to a DEAE-cellulose column. Figure 4 depicts the elution profile of the lytic activity. Two fractions of LT-like material were eluted. The first failed to bind to the DEAE-cellulose under these conditions, while the second broad profile was only slightly bound and eluted shortly after the salt gradient was initiated. If the equilibrating buffer was decreased to 10 mM Tris, no difference was observed in the elution profile. This suggested then that there were two separate activities, and that they

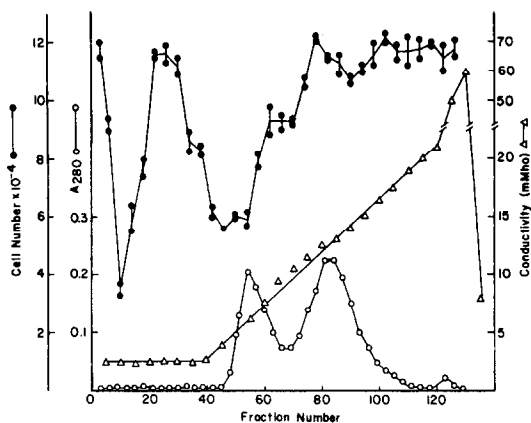


Fig. 4. Elution profile of phosphocellulose bound PGLC-LT off of DEAE-cellulose. The column is equilibrated in 50 mM Tris-HCl, 0.1 mM EDTA (pH 8.0), and the protein eluted with a 0–0.3 M linear gradient of NaCl. Absorbance at 280 nm (\circ), conductivity at room temperature (\triangle) and lymphotoxin activity (\bullet) are plotted vs the fraction number.

were not attributed to a single molecular species of LT whose ability to bind to the column was dependent on the ionic strength of the Tris buffer. In a number of experiments, a third LT activity was observed to elute as a shoulder on the high salt side of the second LT activity. These components of LT which emerge from

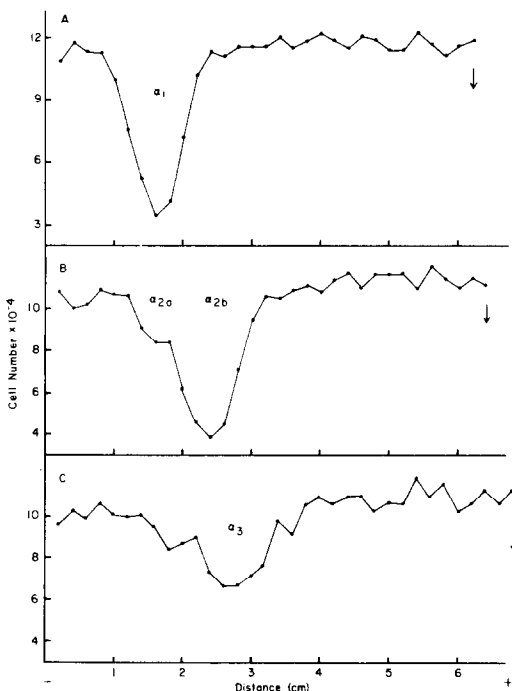


Fig. 5. Profiles of isolated lymphotoxin molecules electrophoresed on 7% polyacrylamide gels. Cytotoxicity was measured with respect to distance of migration. Representative experiments for the unbound LT, α_1 -LT (top), low salt eluting LT, α_2 -LT (middle) and the higher salt eluting LT, α_3 -LT (bottom) from DEAE-cellulose are depicted. Each point represents the mean of duplicate determinations, and the arrows indicate the distance migrated by the marker, Bromphenol Blue.

Table 3. Attempts to detect RNase activity associated with PGLC-LT sub-classes or α -LT sub-classes obtained by fractionation of SAL from PHA activated human lymphocytes^a

Sample added	Quantity	TCA precipitable ¹⁴ C counts in yeast RNA preparation	% control ^b
Control (none)	0	11826	100.0
Pancreatic RNase A	0.05 μ g	1512	12.8
	0.5 μ g	257	2.2
	5.0 μ g ^c	252	2.1
PGLC-LT (α_1)	100 μ l ^e	12447	105.3
PGLC-LT (α_2)	100 μ l	12117	102.4
PGLC-LT (α_3)	100 μ l	12834	108.5
α -LT (α_1)	100 μ l	11845	101.4
α -LT (α_2)	100 μ l	11867	101.2
α -LT (α_3)	100 μ l	11367	96.1

^aTest and control samples were incubated with ¹⁴C labelled yeast RNA. Acid precipitable counts/min were determined as described in text.

^b% of control was determined by
counts/min test
counts/min control

^cEach 100 μ l represents between 200 and 1000 units of LT activity.

the DEAE-cellulose column with increasing salt were consistent with those activities previously defined as α_1 -LT, α_2 -LT and α_3 -LT, respectively, found as sub-classes of α -LT secreted by mitogen activated human lymphocytes *in vitro* (Granger *et al.*, 1978).

Gel electrophoresis

To further characterize the PGLC-LT components resolved on DEAE-cellulose, we subjected the different materials to polyacrylamide gel electrophoresis. As seen in Figs. 5(A), (B) and (C), each of three isolated members of PGLC-LT have their own characteristic relative mobility under these alkaline conditions with respect to the Bromphenol Blue marker. The non-binding α_1 PGLC-LT exhibits a very sharp profile of LT activity with a relative frequency (R_f) of migration of 0.26 [Fig. 5(A)]. The binding α_2 PGLC-LT species shows more heterogeneity with broad migration pattern with an R_f of 0.36 [Fig. 5(B)]. Finally, the shoulder α_3 PGLC-LT component is also diffuse in migration and reveals an R_f of 0.37.

Ribonuclease (RNase) activity

Recently, Lee and Lucas (1976) have reported an RNase activity which copurifies along with the various α -LT components. Because of the important implications of these findings to the mechanism of action of LT on target cells, we undertook a study to determine if there was RNase activity associated with various α sub-classes of PGLC-LT and LT from PHA activated human lymphocytes.

DEAE fractions of PGLC-LT were generated, concentrated, and samples adjusted to contain high levels of LT activity (200–1000 units/ml). In addition, supernatants from PHA stimulated lymphocytes were separated over Sephadex G-150, and the α -LT class further separated into sub-classes on DEAE. Each set of α -LT sub-class was incubated with ¹⁴C- or ³H-labeled yeast or fruit fly RNA as described in the Methods section.

Table 3 depicts a representative experiment

employing ¹⁴C-labeled yeast RNA. It is clear from this study that no RNase activity was detected in separated α -LT sub-classes obtained from either PGLC or mitogen stimulated human lymphocytes. However, positive controls incubated with low levels of pancreatic RNase A showed significant degradation of RNA.

Antigenic properties of PGLC-LT

Previous studies indicated that antisera from rabbits injected with fractions contain all the α -LT sub-classes released by mitogen activated human lymphocytes will neutralize the lytic activity of these molecules *in vitro* (Lewis *et al.*, 1977; Yamamoto *et al.*, 1978). We used preselected antisera (Rabbit A9) which completely neutralizes all α -LT class activity from mitogen activated human lymphoid cells, but does not cross-react with any of the β -LT classes (Lewis *et al.*, 1977). In preliminary studies, we found that antisera to α -LT, but not antisera to β -LT, could neutralize PGLC-LT activity. To further investigate this finding, we decided to physically remove the PGLC-LT-antibody complex, utilizing an indirect antibody precipitation method. One milliliter of PGLC-LT was incubated with 0.1 ml of rabbit anti-human LT or normal rabbit serum overnight at 4°C. This complex was then precipitated with either sheep anti-rabbit serum or goat anti-rabbit IgG after incubation overnight at 4°C. The precipitate was removed by centrifugation, and the supernatants diluted and tested for LT activity. Table 4 indicates that the indirect antibody precipitation removed greater than 90% of the PGLC activity (reduction from 700 units to <5 units). Hence, all the PGLC-LT activity could be removed by antisera specific for α -LT antigenic determinants.

DISCUSSION

Continuous human lymphoid cell lines with characteristics of T and B cells have been previously shown to be capable of releasing lymphokines *in vitro*

Table 4. The capacity of antisera specific for human α -L class activity to neutralize LT activity released by PGLC-33H cell line *in vitro*^a

Experiment	Treatment	LT (units/ml)
1 ^b	Untreated supernatant	235 ± 20
	NRS + sheep anti-rabbit serum	200 ± 27
	Anti- α -LT + sheep anti-rabbit serum	5
2 ^c	Untreated supernatant	89 ± 12
	NRS + sheep anti-rabbit serum	80 ± 15
	Anti- α -LT + goat anti-rabbit rabbit IgG	< 5

^a SAL from PGLC was incubated with anti- α -LT sera and then treated with sheep or goat anti-rabbit serum. The immune complex was removed by sedimentation, and the supernatant tested for LT activity, as described in Materials and Methods.

^b Sheep anti-rabbit serum used to precipitate the PGLC-LT + anti- α -LT complex.

^c Goat anti-rabbit IgG used to precipitate the PGLC-LT + anti- α -LT complex.

(Papageorgiou *et al.*, 1972; Lewis *et al.*, 1977; Yoshida *et al.*, 1976). While the studies were not extensive, physical characteristics of human MIF (Tubergen *et al.*, 1972; Yoshida *et al.*, 1976), chemotactic factor (Yoshida *et al.*, 1976), lymphotoxins (Granger *et al.*, 1970; Papageorgiou *et al.*, 1972; Amino *et al.*, 1974) and interferon (Ware & Granger, in preparation), secreted by continuous lymphoid cell lines, appeared to be generally similar to their counterparts released by mitogen or antigen activated human lymphocytes. Glade and colleagues (Papageorgiou *et al.*, 1972) have repeatedly shown that the human lymphoid cell line, PGLC-33H releases MIF and we demonstrated it releases LT (Granger *et al.*, 1970).

It is clear that LT activity released *in vitro* by mitogen stimulated human lymphoid cells represent a complex 'family of cytotoxins composed of multiple classes and sub-classes of molecules'. The various LT classes are defined by their mol. wt; complex > 150,000 daltons (d), alpha, 70–90,000 d; beta, 30–50,000 d; and gamma, 15–20,000 d (Walker *et al.*, 1976; Hiserodt *et al.*, 1976b; Granger *et al.*, 1978). Also, each LT class contains several sub-classes which can be resolved by ion exchange chromatography and electrophoresis (Walker *et al.*, 1976; Hiserodt *et al.*, 1976a). We undertook our investigation of the cell-toxins released by the continuous B cell lymphoid culture, PGLC-33H, to compare and characterize the heterogeneity of these molecules to those found in supernatant from mitogen activated normal lymphoid cells (SAL).

Initial functional and stability studies suggested PGLC-33H toxins were similar to the stable α -LT class molecules. Cytolytic activity of both types of supernatants was inhibited by 34°C, and there was a certain amount of binding to the target L cells. Also, PGLC-LT was stable at 4°C for long periods and at 56°C, which is a distinct characteristic of the α -LT class (Walker *et al.*, 1976; Hiserodt *et al.*, 1976a; Lee & Lucas 1976). Growth inhibition measured on L-929 or HeLa cells by PGLC supernatants were indistinguishable from SAL, as described previously (Williams & Granger, 1973; Jeffes & Granger, 1976). The growth inhibition exhibited by PGLC supernatants was dependent on concentration

and the particular cell line employed in the assay. However, in contrast to L cells, growth inhibition of HeLa cells by PGLC-LT fractionated over Sephadex G-150 indicated that the effect was not restricted to molecules possessing LT activity, but also possessed by material(s) in the 30–50,000 d mol. wt range. Additional studies have suggested that interferon is probably the active material in these fractions, and this is a species-specific effect (Ware & Granger, in preparation).

The physical characteristics of PGLC-LT appear to be very similar to those seen for the α -LT class released by mitogen activated human lymphocytes. Almost all of the LT activity eluted from Sephadex G-150 is in the 70–90,000 d fraction, characteristic of the α -LT class. Additional separation on ion exchange columns revealed three separable sub-classes of this activity. The majority of PGLC-LT was observed to bind to phosphocellulose during chromatography at pH 6.6 in a linear gradient of NaCl from 0 to 0.3 M. However, on several occasions, a small amount of material not binding to the column was observed. Extensive experiments revealed that this was not due to overloading, because this material was not retained by the resin upon rechromatography under the same conditions. Lee and Lucas (1976) have reported, and we have confirmed, that there is a major LT peak which does not bind to PC columns under these conditions in the α -LT class from mitogen stimulated cells (Granger *et al.*, 1978). It appears that PGLC-33H cells release only small amounts of this sub-class of α -LT activity. The PGLC-LT which was retained by phosphocellulose, and subsequently chromatographed over DEAE-cellulose, exhibited three characteristic sub-class activities observed for α -LT. These three sub-class activities have been defined by their elution order from DEAE-cellulose at pH 8.0 in a linear NaCl gradient of 0–0.3 M. The first, α_1 , does not bind, the second, α_2 , binds weakly and the third, α_3 , binds strongly. Each sub-class, when subjected to separation on PAGE, exhibited somewhat broad but distinctive migration profiles. The PAGE profiles of various α -like LT from PGLC-LT were very similar to those observed for α sub-classes released by lectin

stimulated normal human lymphocytes (Granger *et al.*, 1978). These data would indicate that the PGLC cells are able to release most of the α -LT class of molecules observed in cultures of mitogen activated lymphocytes. The basis of the heterogeneity of the α -LT class of LT activity is not yet clear. Immunologic and physical-chemical studies of these molecules suggest they are similar, and indeed may be isomeric forms of one another, modified by addition of polysaccharide, as reported for a complement of proteins (Meo *et al.*, 1977) and HLA antigens (Parhan *et al.*, 1974).

Other investigators have studied LT-like material(s) from other lymphoid cell lines. Their results suggest that the activity was probably due to member(s) of the 70–90,000 mol. wt α -LT class, but these studies did not describe evidence of sub-class activity. Our finding multiple α -LT sub-classes is due to several reasons: (a) PGLC-33H secretes high enough levels of LT (30–100 units/ml) to permit more extensive physical studies; (b) we employed a sensitive assay system able to detect low levels of activity; and (c) we employed more extensive separation methods.

We found the α -LT class of cytotoxins released by lectin activated human lymphocytes or PGLC-33H cells does not appear to possess RNase activity. This is in contrast to the findings of Lee and Lucas, who reported that all fractions containing α -LT sub-classes possess RNase activity (Lee & Lucas, 1976). None of the various α -LT containing fractions in these studies, obtained from either PGLC-33H or normal lectin stimulated lymphocytes, possessed RNase activity that we could detect. However, positive controls indicated our assay procedure was able to detect less than 50 ng of RNase activity. We have no direct explanation for these conflicting results.

The total abrogation of PGLC-LT activity on target cells *in vitro* by addition of rabbit anti- α class antisera further supports the hypothesis that the LT secreted by this cell line is essentially the same as α -LT released by activated normal human lymphocytes. This was similar to the findings of Amino *et al.* (1974), in that antisera directed toward the LT secreted by a continuous lymphoid cell line could neutralize LT produced from mitogen or antigen activated human lymphocytes.

It is important to consider why supernatants from PGLC-33H only contain α -LT class of molecules. There are several possibilities; those based on the concept that the various classes are unrelated, and the other that they are related forms of one another. Under the former situation, this cell line could represent a variant at the level of LT release, a selective synthesis of only the α classes of LT, or the other soluble phase LT classes are selectively degraded. The latter possibility does not appear to be the case, for mixing experiments revealed that PGLC supernatants do not selectively degrade the complex, β or LT forms. The alternative is that α - and β -LT classes are related, and the PGLC-33H cell line lacks the ability to convert the α to the β -LT form. Physical and immunologic studies indicate this latter possibility may actually be the case (Yamamoto *et al.*, 1978). Additional studies have revealed different populations or sub-populations of human lymphocytes, *i.e.* T or B, are capable of releasing different soluble phase LT classes

when activated with lectin *in vitro* (Harris *et al.*, in preparation). It appears that human T cells can release all classes, but B cells only release α class LT molecules. The present studies suggest that the examination of other human lymphoid cell lines may provide insight into revealing the genetic regulation and control of lymphokines in general.

Acknowledgments—We would like to thank Drs. J. C. Hiserodt, C. F. Ware and L. T. Furbrush for their helpful discussions. We also thank Ms. Margaret Chow for technical assistance and Ms. Gloria Stangl for the preparation of this manuscript.

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