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Authors

Hiserodt, John C
Fair, Daryl S
Granger, GA

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IDENTIFICATION OF MULTIPLE CYTOLYTIC COMPONENTS ASSOCIATED WITH THE β-LT CLASS OF LYMPHOTOXINS RELEASED BY MITOGEN-ACTIVATED HUMAN LYMPHOCYTES IN VITRO[1]

JOHN C. HISERODT, DARYL S. FAIR, AND G. A. GRANGER

From the Department of Molecular Biology and Biochemistry, University of California, Irvine, Irvine, California 92717

Cytotoxic activity(lymphotoxin (LT)) associated with the 50,000 dalton β-LT class of human lymphotoxins has been resolved into multiple components by chromatography on DEAE-cellulose or electrophoresis on polyacrylamide gels. The two components, termed β-LT_1 and β-LT_2, are separable from the 90,000 m.w. α-LT, since neither component is neutralized by incubation with heterologous rabbit anti-α-LT antisera. However, the two components differ in their overall charge and relative heat stability with the βLT_1 component being more positively charged than the β-LT_2 molecule and extremely heat labile.

Recent studies reported from several laboratories have shown that the toxic activity (lymphotoxin (LT)[2]) in supernatants obtained from cultures of mitogen-activated human lymphoid cells (SAL) is due to several macromolecules (1–5). These cytotoxins can be separated by gel filtration into two basic m.w. classes (termed α-LT and β-LT by Walker *et al.* (4)), having characteristic m.w. of 90,000 and 50,000 daltons, respectively. The α-LT cytotoxins are quite stable and have therefore been the major component studied previously by other investigators (6–10). However, β-LT has only recently been identified and is relatively unstable in serum containing medium (4, 11). However, by employing a serum substitute which partially stabilizes β-LT (11), we have been able to resolve this cytotoxin into at least two components (termed β-LT_1 and β-LT_2), which are separable by chromatography on DEAE or electrophoresis on polyacrylamide gels.

MATERIALS AND METHODS

Target cells and culture media. Target cells were obtained from stock cultures of mouse L-929 fibroblasts maintained in RPMI 1640 (Grand Island Biological Co., Grand Island, N.Y.), supplemented with 3% heat-inactivated (56°C, 1 hr) fetal calf serum, streptomycin, and penicillin. These cells were grown at 37°C in 32-oz prescription bottles in 95% air, 5% CO_2, and passed biweekly.

Production of human LT. The details of these methods have been described previously (12, 13). Briefly, human small lymphocytes were obtained from tonsils or adenoids of patients 6 to 8 hr after surgical removal. Cultures were established in RPMI 1640, supplemented with streptomycin (100 μg/ml), penicillin (100 units/ml), and 20 μg/ml of a heat stable bovine serum fraction containing 4×10^6 viable cells/ml in 32-oz prescription bottles. Activation of the lymphocytes was effected with phytohemagglutinin-P (PHA-P, Difco, Detroit, Mich.) at 20 μg/ml and culturing for 5 days at 37°C in 95% air, 5% CO_2. Supernatants were cleared of cells by centrifugation, filtered, and stored at −20°C. The SAL were pooled and concentrated 50 times by passage through a Bio-Rad Hollow Fiber Concentrator with a filter retaining molecules greater than 30,000 m.w., and stored at −20°C until used.

Physico-chemical separation of LT. The details of the methods employed for fractionation of LT activity in supernatants from activated lymphocytes have been previously published (7). However, each is described briefly:

A. Sephadex chromatography. Sephadex G-100 (Pharmacia, Upsala, Sweden) was poured in a 2.5- x 120-cm siliclad coated column to a bed height of 100 cm and equilibrated in 0.02 M phosphate buffer, pH 7.2, 10^{-4} M EDTA. Calibration was effected with various m.w. markers: blue dextran (B.D.) 2×10^6 daltons), bovine serum albumin (BSA) (68,000), ovalbumin (Oval) (44,000), cytochrome *c* (Cyt. *c*) (12,500), and phenol red (P.R.) (354) applied in a total volume of 2.0 ml. Fractions were collected (5.0 ml) and monitored for their absorbance at 280 nm on a Gilson Model 2000 spectrophotometer, (Middleton, Wis.).

B. DEAE-cellulose chromatography. DEAE (DEAE 11, Whatman) was poured to a bed height of 12 cm in a 2.5 x 20 cm siliclad-coated column and equilibrated in 50 mM Tris HCl, pH 8.2, 10^{-4} M EDTA (starting buffer). A sample (4 ml) of β-LT obtained after rechromatography on Sephadex G-100 was dialyzed against starting buffer and applied to the column. Fractions were eluted in a linear salt gradient from 0 to 0.35 M NaCl in the same starting buffer. Fractions (5.0 ml) were collected and tested for conductivity and LT activity.

C. Polyacrylamide disc gel electrophoresis. PAGE was performed on fractions of β-LT obtained after rechromatography on Sephadex G-100. A 100-μl sample was applied to a 0.5- x 8.0-cm gel (7% acrylamide) with a 1-cm stacking gel in 50 mM Tris-glycine, pH 9.6. The sample was subjected to electrophoresis at 4 mA/gel for 2 hr. The gels were then cut into 2-mm slices, and each slice was incubated in 0.3 ml RPMI 1640, 3% FCS for 24 hr at 4°C. The medium was then tested in duplicate for LT activity.

LT assays. The details of these methods have been reported previously (14). Briefly, 10^5 L cells in 1.0 ml were established as monolayers in screw-capped tubes in RPMI 1640, 3% FCS containing 0.5 μg/ml mitomycin C. After a 24-hr incubation at

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[2] Abbreviations used in this paper: LT, lymphotoxin; SAL, supernatants from cultures of mitogen-activated human lymphoid cells; B.D., blue dextran; oval, ovalbumin; Cyt. *c*, cytochrome *c*; P.R., henol red.

37°C, the medium was discarded and serial dilutions of LT containing or control media were added to duplicate cultures, and they were incubated for 24 hr at 37°C. The cell number was then enumerated on a Model F Coulter Counter. Units of LT activity are expressed as the reciprocal of the dilution of LT necessary to kill 50% of the target L cells.

Column fractions were assayed for LT activity by adding 0.05 or 0.10 ml to 1.0 ml of a preestablished monolayer of L cells (10^6) and allowing them to incubate for 24 hr at 37°C. The remaining viable adherent cell numbers were then enumerated on a Coulter Counter identical to the procedure described above.

Antibody neutralization. The methods for obtaining and testing specific antisera have been reported by Lewis, *et al.* (15). Briefly, New Zealand White Rabbits were immunized with fractions of α-LT purified by various methods and suspended in complete Freund's adjuvant. Serum was collected after multiple injections, heat inactivated 56°, 60 min, and tested. Neutralizations were performed by incubating antisera (200 μl) or normal rabbit sera (200 μl) with 1 ml of LT containing media for 1 hr at 37°C, after which it was diluted and tested for activity (units/ml) as described above.

RESULTS

Separation of the α-LT and β-LT cytotoxins in SAL by gel filtration chromatography on Sephadex G-100. Cytotoxic activities associated with α-LT and β-LT, as previously reported by Walker, *et al.* (4), were physically separated by gel filtration chromatography employing Sephadex G-100. Shown in Figure 1*A* is the elution profile of 2.0 ml of a 5 day 50 times concentrated SAL. The elution profile of the m.w. markers employing blue dextran, BSA, ovalbumin, and Cyt. *c* is indicated by vertical arrows. The α-LT and β-LT peaks overlap considerably, however, α-LT activity eluting in front of the BSA marker and β-LT activity eluting close to the ovalbumin marker were further resolved by rechromatography on similar columns. Fractions containing β-LT activity were pooled (shown) and concentrated by ultrafiltration (Amicon PM10 Ultrafilter) to 2.0 ml and rechromatographed on the same G-100 column. As shown in Figure 1*B*, β-LT is completely resolved from α-LT by this procedure. The small contaminating peak of α-LT activity seen accounts for 5 to 10% of the total activity originally applied to the column.

Resolution of the α and β-LT into multiple components by DEAE cellulose chromatography. Fractions containing β-LT activity, after rechromatography on Sephadex G-100, were pooled, concentrated, dialyzed by ultrafiltration against starting buffer to 4.0 ml, and layered on a 2.5 x 12 cm DEAE-cellulose (DEAE 11, Whatman) column pre-equilibrated in the same buffer. The column was then washed with 100 ml of starting buffer and a 400 ml NaCl linear gradient (0.0 M to 0.35 M NaCl) was applied. Fractions were collected (5.0 ml) and tested for LT activity as described in *Methods*. The DEAE elution profile is shown in Figure 2. Peak 1 elutes immediately off the column with 0.0 M NaCl at pH 8.2. However, a rather broad second peak elutes off at a salt concentration of 0.12 M NaCl. These activities have been termed β-LT_1 and β-LT_2, respectively. These identical results were obtained in two separate experiments.

Resolution of β-LT into multiple components by PAGE. In order to substantiate further the results observed with DEAE, rechromatographed Sephadex fractions containing peak β-LT activity were subjected to PAGE (7% acrylamide) in 50 mM Tris-glycine, pH 9.6. A 100-μl sample was applied to a 0.5- x 8.0-cm gel and subjected to electrophoresis at 4 mA/gel for 2 hr. The gel was cut into 2-mm slices, and each slice was eluted in 0.3 ml of RPMI 1640 - 3% FCS for 24 hr at 4°C. The media was tested for LT activity as described in *Methods*. The results shown in Figure 3 are typical of several experiments and indicate that β-LT can be separated into two distinct peaks of activity with R_f values of 0.28 and 0.46 relative to the migration of the bromphenol blue marker.

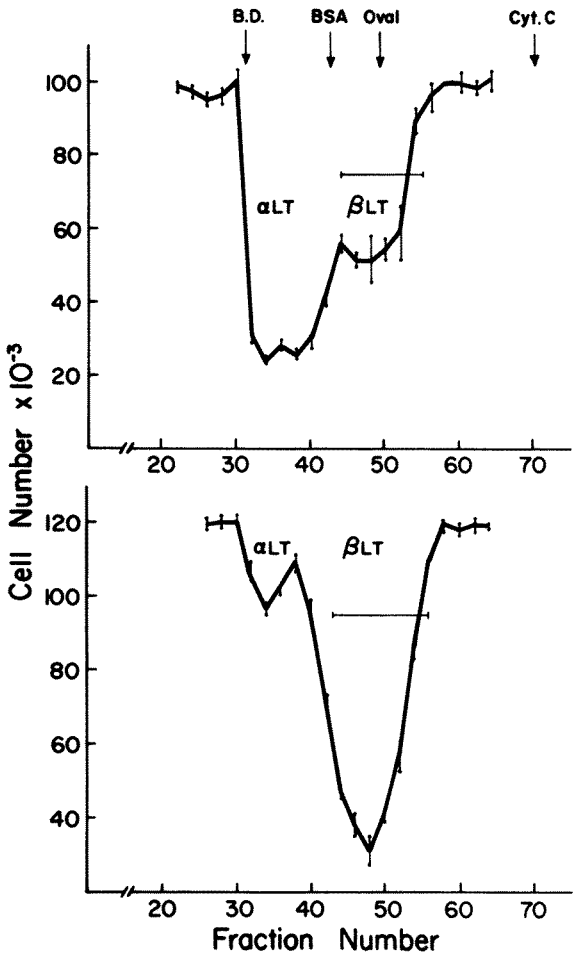

Figure 1. Chromatography of 5-day human SAL on Sephadex G-100. *Upper*, a 2.0-ml sample of a 5-day 50× concentrated SAL was chromatographed over a 2.5- x 120-cm Sephadex G-100 column. Fractions were collected and 0.1 ml was tested for LT activity as described in *Materials and Methods*. The abscissa shows the remaining visible cells after a 24-hr incubation, and the ordinate indicates column fractions tested for LT activity. The elution pattern of various molecular weight markers (which are abbreviated as described in *Materials and Methods*) is indicated by vertical arrows. Vertical lines represent errors in cell counts in duplicate tubes. Horizontal bar indicates β-LT fractions taken for rechromatography. *Lower*, Elution profile of the β-LT rich fractions after rechromatography on Sephadex G-100. The β-LT rich fractions (tubes 43 to 56) shown in upper figure were pooled, concentrated to 2.0 ml (PM10), and applied to the same G-100 column. Horizontal bar represents β-LT fractions taken for chromatography on DEAE-cellulose.

Comparison of some of the physical properties of human β-LT_1 and β-LT_2. *In vitro* cytotoxic activities associated with human α-LT and β-LT were compared for heat stability and neutralization by rabbit anti-α-LT antisera. As shown in Table I, α-LT is stable to heating at 56°C for 4 hr. It can also be totally neutralized (> 98%) by incubation with rabbit antihu-

man α-LT antisera (15). The β-LT cytotoxins, however, are not neutralized by anti-α-LT antisera but show a significant decrease in activity when incubated at 56°C for 4 hr (60% loss). This loss of activity during heating was shown by testing individual β-LT components to be due to the extreme heat lability of the β-LT_1 component. The β-LT_2 component, however, does not appear to be greatly affected by similar heat treatment. Recent studies have shown that when the β-LT_1 and β-LT_2 components obtained from DEAE are placed on polyacrylamide gels, the β-LT_1 component migrates as a single peak with an R_f value of 0.28, whereas, the β-LT_2 component migrates as a wide peak having an R_f value of 0.33 to 0.39. This suggests that there may be as yet an unresolved third component of the β-LT_2 class of cytotoxins.

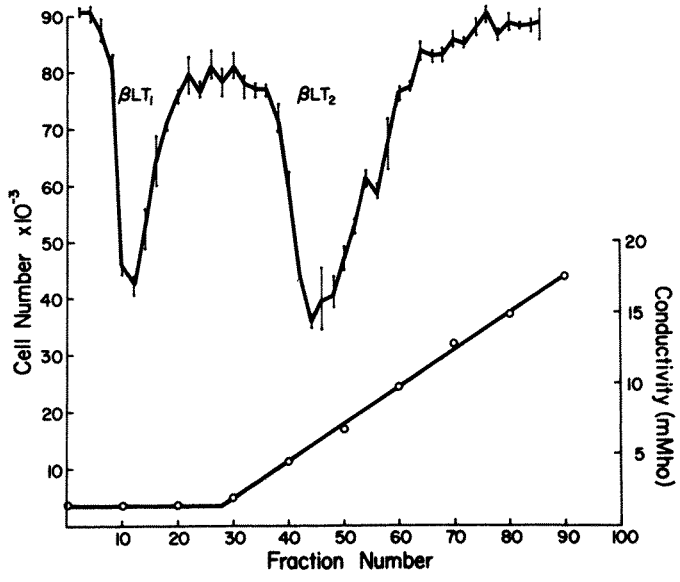

Figure 2. Chromatography of β-LT released by human lymphocytes *in vitro* on DEAE-cellulose. The β-LT fractions obtained after rechromatography on Sephadex G-100 were concentrated, dialyzed against DEAE starting buffer, and eluted with a 400-ml linear NaCl gradient (0 to 0.35 M) in the same buffer. Fractions were collected and tested for conductivity and LT activity as described in *Materials and Methods.*

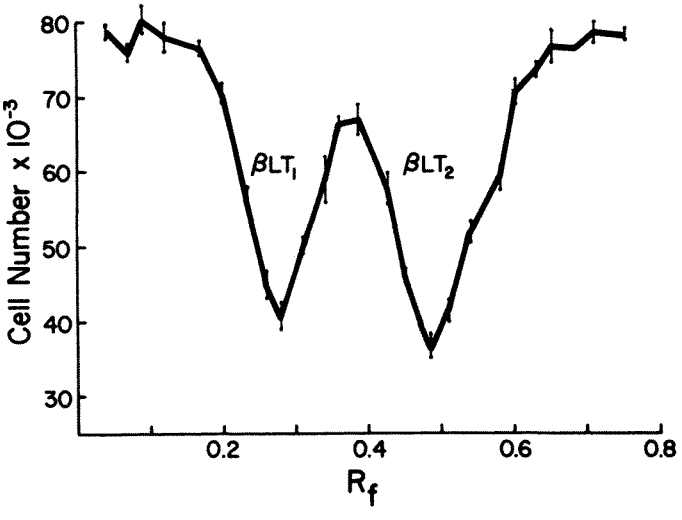

Figure 3. Polyacrylamide disc gel electrophoresis of human β-LT activity. An aliquot of the same sample which was applied to the DEAE column was also subjected to PAGE. A 100-μl sample was electrophoresed on 7% polyacrylamide gels (pH 9.6), the gels were sliced, and the LT activity was eluted and tested as described in *Materials and Methods.* R_f values are those relative to the migration of the bromphenol blue marker.

TABLE I

Comparison of certain physical properties of the α and β class of LT molecules released by PHA-activated human lymphocytes in vitro[a]

Species of LT	Ab neutralization		Heat stability (56° 4 hr)
	NRS (control)	Anti α-LT	
α-LT	1400 ± 70	20 ± 3	1340 ± 50
β-LT	120 ± 10	100 ± 13	52 ± 3
β-LT_1	10 ± 2	8 ± 1	No activity
β-LT_2	50 ± 5	45 ± 3	43 ± 2

[a] Human α and β lymphotoxins were obtained by chromatography of 5 day SAL on Sephadex G-100. Primary separation yielded fractions containing α-LT, whereas rechromatography of the β-LT rich fractions on the same G-100 column yielded β-LT. The β-LT fractions obtained after rechromatography were then pooled, dialyzed, and passed over a DEAE-cellulose column to obtain the β-LT_1 and β-LT_2 activties. Each LT activity was then subjected to heating at 56°C for 4 hr or neutralization by rabbit anti-α-LT antisera, as described in *Materials and Methods.* Results are expressed as the units of LT activity ± standard error.

DISCUSSION

These studies have shown that the cytotoxic activity associated with the β-LT class of LT molecules released by PHA-activated human lymphocytes *in vitro* consists of multiple components. These components appear to have the same m.w. based on Sephadex chromatography (50,000 daltons) as reported by Walker *et al.* (4), but differ in charge and can be resolved, therefore, by chromatography on DEAE-cellulose or electrophoresis on polyacrylamide gels. The two components have been termed β-LT_1 and β-LT_2, respectively, with the β_1 molecule being less charged than the β_2 molecule. The heat stability of the two components suggest that although the β_1 molecule is very heat labile at 56°C, the β_2 molecule is relatively heat stable. However, neither component can be neutralized by rabbit anti-α-LT antisera (15). This is in contrast to the 90,000 dalton α-LT molecule(s), which are totally neutralized by incubation with anti-α-LT antisera and are very heat stable at 56°C.

The actual number of β-LT cytotoxins has not yet been clearly established. Our studies have shown that the β_2 component elutes as a wide peak of activity off both DEAE and PAGE. This would suggest that there may be another component(s) associated with β_2 activity. The β_1 component, however, elutes as a sharp single peak off both DEAE and PAGE, suggesting that it is homogeneous. It is important to note, however, that the stability of these cytotoxins dictates rapid fractionation and assay procedure, since the half-life of the β_1 molecules can be as short as a few hours at 37 or 4°C in serum-containing medium. However, these molecules are relatively more stable in cultures supplemented with our serum substitute (11).

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