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THE HUMAN LT SYSTEM

XII. Purification and Functional Studies of LT and "TNF-like" LT Forms from a Continuous Human T Cell Line[1]

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A clone of the continuous human T cell line HUT-102, termed YM 1.2, can spontaneously release α-LT in vitro. However, when stimulated with phorbol myristic acetate, these cells release other LT forms. These LT forms were purified to homogeneity by DEAE chromatography, column isoelectric focusing, and polyacrylamide gel electrophoresis. One LT form, termed LT-2, is a 79,000 m.w. component in aqueous solution and composed of 21,000 m.w. subunits. This form is immunologically related to macrophage-derived TNF and has a lytic capacity in vitro on K-562, Molt-4F, and Raji cells similar to that described for cytotoxins derived from NK effector cells, termed NK-CF. A second LT form, termed LT-3, is a single 69,000 m.w. peptide which could not be reduced into the smaller subunits. This form expresses antigens in common with both α-LT and TNF, because both anti-LT and anti-TNF were required to completely neutralize cell lytic activity in vitro. Functional testing revealed that the LT-3 form is lytic on all continuous cells tested in vitro, including NK-resistant target cells. The LT-3 component appears similar by immunologic, biochemical, and functional criteria to the LT form derived from primary human cytolytic T cells in vitro. At the levels tested, none of these LT-TNF forms had measurable effects on primary fibroblasts in vitro.

Activated lymphocytes and macrophages from humans and experimental animals can be stimulated in vitro to release materials that are growth-inhibitory and lytic for transformed cells in vitro (1, 2) and have anti-tumor effects in vivo (3–5). Materials with in vitro cell lytic activities were initially termed lymphotoxins (LT)[3] and macrophage cell toxins (MCT), respectively (2, 6–8), and materials active against tumors in vivo were termed tumor necrosis factors (TNF) (5). Recent biochemical evidence shows that one human LT form, termed α, and one TNF form are distinct proteins; however, they are related because they share a 36% amino acid sequence identity and 51% homology (9). Both lymphocyte- and macrophage-derived effector molecules can cause TNF effects in mice, and thus LT, MCT, and TNF are functionally and physically interrelated. It is generally accepted that MCT-TNF is released by macrophages and that α-LT is released by lymphocytes (6, 10). For ease of understanding, we will refer to the MCT-TNF form as TNF and not refer to LT as TNF. Previous studies demonstrated that LT forms in supernatants from stimulated unseparated human lymphocytes are heterogeneous (1). This led to the concept that different populations of effector cells may be capable of releasing LT forms with different functional capabilities (1, 11). This premise was supported by the discovery of cell lytic forms, termed NK-CF and NK-LT, that are immunologically distinct from α-LT, derived from natural killer (NK) effector cells, and more effective on NK-sensitive than on NK-resistant target cells in vitro (12, 13).

The preceding companion paper in this series (11) demonstrated that nonspecific and specific human cytotoxic T cells (CTL) can be stimulated by lectins or contact with target cells to release multiple LT forms. One form was shown to be α-LT, and a second form was distinguished from α-LT by immunologic, biochemical, and functional parameters. The second form did not appear to be NK-LT because it lysed target cells which have been shown to be resistant to NK-LT action in vitro (11). Immunologic studies revealed that the new LT form(s) was neutralized by a combination of anti-α-LT and anti-TNF serum. Unfortunately, these studies revealed that primary CTL lines would not provide the quantities of material for biochemical studies (11).

The present report identifies a continuous human T cell line, termed YM 1.2, which can be stimulated with phorbal myristic acetate (PMA) to release high levels of cell toxins. Three active materials released by YM 1.2 cells have been identified, and two were purified to homogeneity. Each purified form expresses a unique cell lytic ability when tested on a panel of transformed cells, but has no effect on primary cells in vitro. The first form is α-LT; a second form, termed LT-2, appears immunologically and biochemically similar to TNF; and a third form is distinct biochemically and functionally from

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[1] This research was supported by Rheumatic Diseases Research Foundation Grant 1882 and Fujisawa Pharmaceutical Co., Ltd.
[2] To whom correspondence should be addressed.
[3] Abbreviations used in this paper: LT, lymphotoxins; MCT, macrophage cell toxins; TNF, tumor necrosis factors; CTL, cytotoxic T cells; NRS, normal rabbit serum, IEF, isoelectric focusing; rTNF, recombinant human TNF.

these two LT forms, but shares immunologic determinants with both α-LT and TNF.

MATERIALS AND METHODS

Cell cultures and cell lines. Continuous human lymphoid cell lines were maintained as suspension cultures in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS) (GIBCO) (RPMI-10%) as described (11). Human T cell lines were obtained from Dr. J. Minowada, Roswell Park Memorial Institute, Buffalo, NY. The human B cell line GM3104A was obtained from the Human Gene Mutant Repository, Camden, NJ. Non-lymphoid continuous and normal human and murine cell lines were grown as monolayer cultures in Corning T-75-cm^2 tissue culture flasks in RPMI-10%, except the L-929 cell, which was grown in RPMI 1640 supplemented with 3% FCS. These continuous monolayer cultures were passed biweekly, and normal cell cultures were passed when they reached confluency.

Cell lytic assays. All assays for monolayer cultures were conducted in 96-well, flat-bottomed microtiter plates with 10,000 to 20,000 target cells/well in 100 μl of medium as described (11). In brief, 10,000 to 20,000 target cells/well were established 24 hr before the addition of various dilutions of sample. After incubation with the sample, the medium was aspirated off, the remaining adherent target cells were stained with crystal violet, and the amount of stain in each well was measured in a Flow Titertek Multiskan Plate Reader. The assay for nonadherent cells was performed by using 96-well, round-bottomed microtiter plates as described (14). Different dilutions of sample were added to target cell monolayers (10,000 cells/well) in microplates. After incubation, the cells were treated with the vital dye MTT (Sigma Chemical Co., St. Louis, MO), were fixed with glutaraldehyde, were washed, and were solubilized. The amount of stain in each well was measured on the Plate Reader. The amount of stain in these wells is directly proportional to the number of cells present. The effect of a test material is derived by comparison of cell number in the test and control wells. All assays were performed in triplicate. Two microplate assays for LT which employ L-929 target cells were also used; one detects the presence of LT activity, the other measures the amount of LT activity in U/ml in a sample. Each method has been described (15). A unit of LT activity is that amount of material which lyses 50% of 15,000 to 20,000 nondividing L-929 target cells in a 16- to 24-hr period (16).

Supernatant production. A) Small-scale cultures. Human T cells were maintained in Corning T-25-cm^2 flasks until they reached a cell density of 1.5×10^6 cells/ml. They were then transferred into fresh medium at a cell density of 2.5×10^5 cells/ml and were cultured in the presence or absence of 20 ng/ml of PMA at 37°C. After 72 hr, supernatants were collected, were cleared of cells by centrifugation at 4000 × G for 15 min, and the samples were then tested immediately for LT activity or were frozen at −70°C. B) Large-scale culture. The YM 1.2 cells were grown to a density of 1.5×10^6 cells/ml in RPMI-10% in 3-L roller bottles. These cells were then washed with serum-free RPMI 1640 (RPMI-0%) by alternate sedimentation at 450 × G for 5 min and resuspension in RPMI-0%. After the fourth wash, the cells were resuspended in RPMI-0% at a cell density of 2.5×10^5 with 20 ng/ml PMA, and 800 ml were cultured in 3-L roller bottles. After 24, 48, or 72 hr at 37°C, the supernatants were collected, the cells were removed by sedimentation at 4000 × G for 15 min, and the number of lytic units was determined. Although the results are not shown, they revealed that PMA induced release of higher levels of cell lytic activity by 24 hr which remains relatively unchanged to 72 hr. Supernatants were pooled into 5-L lots, were passed through a 0.2-μm filter, and were concentrated 70-fold by ultrafiltration in an Amicon Stirred Cell (Amicon, Danvers, MA) with a YM-10 membrane. The concentrates were centrifuged for 2 hr at 110,000 × G in a Beckman Model L3-40 ultracentrifuge, were passed through a 0.05-μm filter, and were stored at −70°C.

Molecular sieving. Supernatant samples were subjected to molecular sieving on 2.5 x 100-cm Ultrogel AcA 44 (LKB, Gaithersburg, MD) columns equilibrated in 0.01 M phosphate, 0.1 mM EDTA, pH 6.9, at 4°C as described in detail (15). Samples of 1 ml were loaded and 6-ml fractions were collected at a flow rate of 24 ml/hr. The column was calibrated with thyroglobulin (660,000 m.w.), human IgG (150,000 m.w.), hemoglobin (64,000 m.w.), α-chymotrypsinogen (23,000 m.w.), and phenol red (395 m.w.). Each fraction was analyzed for protein and LT activity.

DEAE ion-exchange chromatography. Concentrated samples were subjected to ion-exchange chromatography on DEAE as described in detail (15). Seventy-fold concentrates were dialyzed against 1000 vol of 0.01 M Tris, pH 8. These concentrates were bound to and eluted from a 2 x 10-cm DEAE column with a 0.0 to 0.3 M NaCl gradient in 0.01 M Tris, pH 8, buffer. Concentrates of 5-L lots were applied and were eluted with a 120-ml gradient into 3-ml fractions at a flow rate of 30 ml/hr. Protein, bioactivity, and conductivity were assessed for each fraction. The pH of each sample was adjusted to 7 with 0.01 M Tris, and either 1 mg/ml bovine serum albumin (BSA) or 5% polyethylene glycol was added to stabilize biologic activity.

Column isoelectric focusing (IEF). Fractions containing the lytic activity from the DEAE column were pooled and were subjected to IEF in a 110-ml LKB IEF column in a 5 to 8 or 4 to 8 pH gradient in 3% ampholines as described (17). In brief, focusing columns were run at 700 V under constant voltage for 14 hr or until they reached equilibrium. The columns was eluted at a flow rate of 20 ml/hr into a fraction volume of 1.8 ml. Individual fractions were assayed for bioactivity and pH.

Polyacrylamide gel electrophoresis (PAGE). The discontinuous gel system of Davis (18) was employed in these studies. Application, separation, and elution of 100- to 200-μl LT samples on native tube (0.5 x 8-cm) PAGE tube cells has been described in detail elsewhere (17). The Rf values were calculated with reference to the migration of a bromphenol blue marker. PAGE gels, 1-cm stacking and 8-cm running, were poured in slab gel plates employed for SDS gels described below. Samples of 20 to 30 μl were loaded and were electrophoresed at 20 mA/gel at 4°C. Gels were stained with silver by the method of Merril et al. (19). Individual vertical gel lanes were sliced out and were then cut into 2-mm horizontal slices; individual slices were incubated with 300 μl RPMI-10% overnight at 4°C. Various dilutions of each supernatant were tested on L-929 cells for bioactivity.

Polyacrylamide SDS slab gel electrophoresis. Samples were subjected to electrophoresis in SDS nonreducing or reducing (0.1% mercaptoethanol) slab gels by the technique of Laemmli (20). Samples were run in 0.1 x 20 x 20-cm slab gels, each overlaid with a 1- to 1.5-cm stacking gel at 20 mA/gel at 4°C. The slab gels were silver stained by the method of Merril et al. (19). Vertical gel lanes were sliced into horizontal 2-mm fragments. Individual fragments were eluted with 300 μl of RPMI-0% for 24 hr at 4°C. The supernatant was then dialyzed against 500 vol of RPMI-0% for 24 hr before being assayed on L-929 cells for lytic activity. The position of bioactivity and protein in these gels were compared to known m.w. markers as indicated in the figures shown with each gel.

Preparation and use of antisera. Antiserum from rabbits immunized with pure α-LT from GM3104A lymphoblastoid cells which neutralizes LT activity in vitro has been described (21, 22). Two different antisera from rabbits were also employed: A) serum from animals immunized with recombinant human TNF (rTNF, a gift from Cetus Corp., Emeryville, CA); and B) serum generated against partially purified MCT from PMA-stimulated human promyelocyte cell line ML-2, a gift from Dr. G. Gifford, University of Florida College of Medicine (23). Purified rTNF and monoclonal antibody prepared against rTNF was a gift from Biogen Research Corp. (Cambridge, MA). Supernatant-containing human TNF was obtained from PMA-stimulated THP-1-0 cells, a monocytic cell line, as described (8). The levels of bioactivity in all samples were pretested and were adjusted to 5 U/ml. The in vitro neutralizing capacity of each immunologic reagent to neutralize their respective factors was pretested and was standardized. This was accomplished by testing serial dilutions of antibody against 5 U of cell lytic activity in vitro on L-929 cells in the microplate assay. All experiments were repeated a total of three times, and the percent neutralization of lytic activity was determined by the following formula:

$$\frac{(\text{L-929} + \text{test} + \text{antibody}) - (\text{L-929} + \text{test} + \text{control serum})}{(\text{L-929} + \text{control serum}) - (\text{L-929} + \text{test} + \text{control serum})} \times 100$$

RESULTS

Release of cell lytic materials by PMA-stimulated continuous human T cell lines in vitro. Various T cell lines, CCRF-HSB-2, HD-Mar 2, HPB-All, HPB-MLT, HUT-78, HUT-102, JM, KE-37, KOPT-KI, MOLT-3, MOLT-4, MT-1, Peer, P30/OKUBO, RPMI-8402, SKW-3, TALL, and YT-4E, were cultured in the presence or absence of PMA as described in *Materials and Methods.* The results of these studies indicate that two human T cell lines, HUT-102 and YT-4E, spontaneously release material(s) with LT-like activity. However, when cultured with PMA, the supernatant from these cell lines contains much higher levels of bioactivity. The PMA also induced KE-37 cells

to release LT-like materials. Additional tests revealed that these cells did not produce high levels of LT when incubated with various lectins. The HUT-102 cells were chosen for further studies because they are high producers of cell lytic material(s). The cells from this line were cloned on a monolayer of BALB/c peritoneal macrophages in 96-well, flat-bottomed microtiter plates by the limiting dilution method of Levey et al. (24). Five subclones were isolated by repeated cloning and selection of high level producers. All studies reported here were conducted with clone YM 1.2. This clone routinely produces from 400 to 550 U of activity/ml when stimulated with PMA. Subsequent testing of stimulated supernatants on 15 different cell lines revealed that activity was not neutralized by anti-LT serum and was lytic for a panel of continuous cells, including those resistant to NK-LT. We decided to further characterize the active materials in the PMA-induced supernatants from YM 1.2 cells.

PMA stimulation induces YM 1.2 cells to release new LT forms. The PMA-induced and noninduced YM 1.2 supernatants were concentrated and were subjected to gel filtration chromatography. Shown in Figure 1 (open circles) is the elution profile of a 48-hr supernatant from nonstimulated YM 1.2 cells. It is apparent that cell lytic activity is resolved into one major peak of 80,000 to 100,000 m.w. and a shoulder of activity at 65,000 to 75,000 m.w. Also shown in Figure 1 (closed circles) is an elution profile of a 48-hr supernatant from PMA-stimulated YM 1.2 cells. There is a single peak of cell lytic activity in the 65,000 to 75,000 m.w. area with little evidence of the 80,000 to 100,000 m.w. material which predominates in the unstimulated supernatant. Further testing revealed that the in vitro lytic activity of the 65,000 to 75,000 m.w. material was not neutralized by anti-LT serum. Similar results were obtained when 24- and 72-hr supernatants were chromatographed on these same columns. Identical results were also obtained when these same fractions were tested on HeLa cells; however, the data in Figure 1 employed L-929 cells as targets.

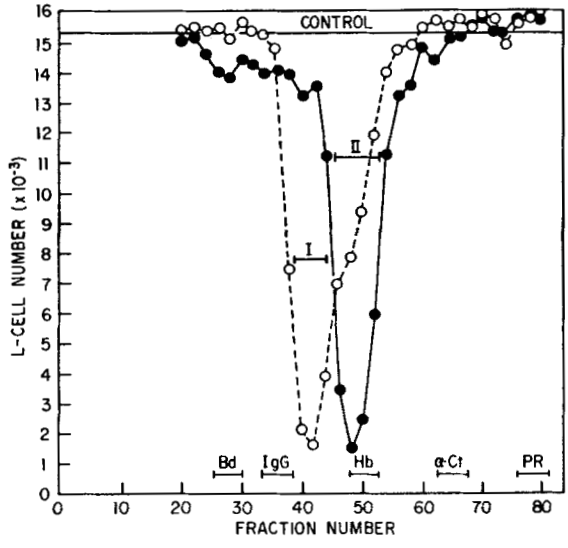

Figure 1. Cytotoxic elution profile of 48-hr supernatant from unstimulated (○) and stimulated (●) YM 1.2 cells on an AcA 44 molecular sieving column. The elution profiles of various m.w. markers are indicated by the horizontal bars: blue dextran (Bd >200,000 m.w.), human IgG (150,000 m.w.), hemoglobin (Hb 64,000 m.w.), α-chymotrypsin (α-Ct 23,000 m.w.), and phenol red (PR 395 m.w.).

Fractionation of YM 1.2 supernatants on DEAE and IEF column. Seventy-fold concentrates of 5-L supernatant lots from PMA-stimulated YM 1.2 cells were first chromatographed over DEAE columns as described in *Materials and Methods.* The profile of one of these columns is shown in Figure 2. Cell lytic activity elutes from this column as a single peak of activity. The fractions containing the major activity were pooled as indicated by the bar in Figure 2, were concentrated to 10 ml by ultrafiltration, were protein-quantitated, and the total units of activity in the pool were determined. The sample was dialyzed and was subjected to column IEF. The results from the pH 5 to 6 region of an IEF column are shown in Figure 3. Cell lytic activity separates into two major peaks which focus at pH 5 to 5.4 and pH 5.6 to 6. In certain lots, a peak is also seen at pH 6.3. Biochemical and immunologic studies revealed that the pH 6.3 peak is α-LT; in addition, in some of the IEF columns, some α-LT activity was also detected at the pH 5.8 fraction.

Purification of two LT forms from the IEF columns. One hundred microliters of the material from each major peak were subjected to electrophoresis in PAGE tube gels as described in *Materials and Methods.* The pH 5 to 5.4 material migrates as a single sharp symmetrical peak with an Rf of 0.41, whereas the pH 5.6 to 6 material migrates as a single sharp peak with an Rf of 0.38. The samples eluted from tube gels were then rerun on native PAGE slab gels. The slab gel was cut in between transverse lanes of migration; one lane was silver stained, and the other lane was cut into 2-mm slices and was tested for bioactivity. The results are shown in Figure 4*A* and *B*, respectively. Comparison of silver-stained gels and the position of bioactivity show a single protein band that coincides with bioactivity. Materials with an isoelectric point of 5.6 to 6 and 5 to 5.4 were termed LT-2 and LT-3, respectively.

Purification of the LT-2 and -3 forms. A summary of the results of a complete purification run are shown in Table I. There is an apparent yield of 1 to 5% of the starting material. By comparing intensity of stained LT bands to stained protein standards, we calculate a specific activity of 10^7 U/mg for both the pH 5 to 5.4 (LT-3) and pH 5.6 to 6 (LT-2) materials.

Analysis of LT-2 and LT-3 in SDS reducing and nonreducing gels. The LT-2 and LT-3 eluted from the native PAGE tube gels were subjected to electrophoresis in SDS nonreducing slab gels. The results shown in Figure 5 indicate that LT-3 (Fig. 5*A*) and LT-2 (Fig. 5*B*) migrate as single bands of 69,000 ± 1000 m.w. and 79,000 ± 2000 m.w., respectively, in nonreducing gels. Additional studies were conducted to attempt to determine whether bioactivity migrates with the protein bands detected in these gels. Both the LT-3 and LT-2 material from PAGE gels were run in nonreducing SDS gels, the gels were sliced, materials in slices were eluted, and the eluate was dialyzed and was tested for bioactivity as described in *Materials and Methods.* The results shown in Figure 5 indicate that the biologic activity recovered from these gels coincides with the stained band of protein. The PAGE samples of LT-2 and -3 were then subjected to electrophoresis in SDS reducing slab gels and the gels were silver stained. The data shown in Figure 6 indicate that the LT-3 (Fig. 6*A*) appears to be a single band at 69,000

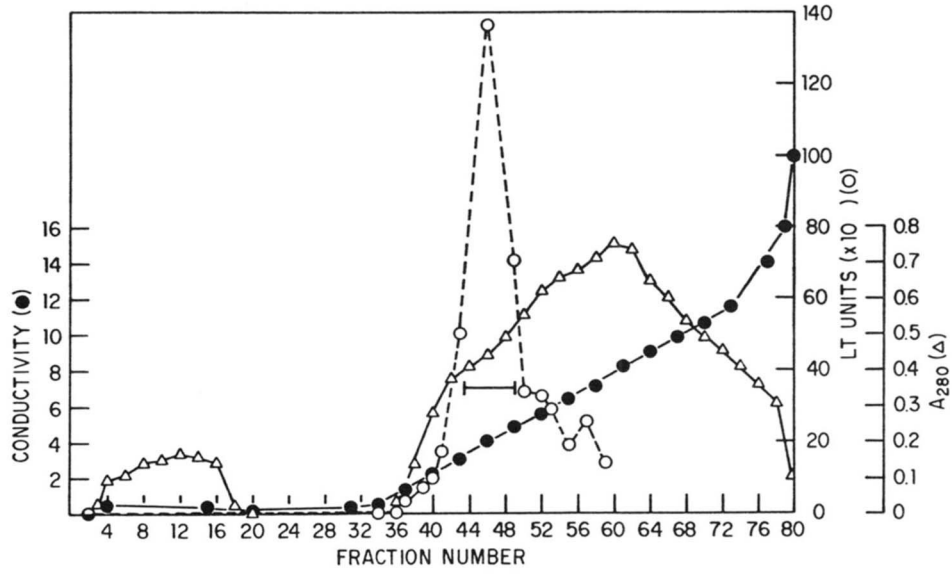

Figure 2. DEAE cellulose chromatography of 70 x YM 1.2 concentrate. PMA-stimulated YM 1.2 cultures were concentrated and were dialyzed against DEAE starting buffer. The sample was applied to the column and was eluted with a 120-ml, 0 to 0.3 M NaCl gradient, in 0.01 M Tris, pH 8. Fractions were tested for conductivity (●), bioactivity (○), and protein (△).

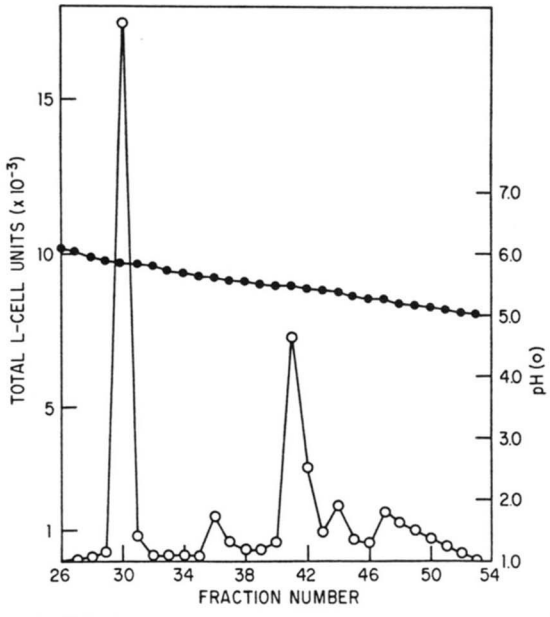

Figure 3. IEF of YM 1.2 lytic activity pooled from a DEAE cellulose column. The sample was pooled as shown by the bars in Figure 2, was concentrated, was dialyzed against 0.01 M Tris buffer, and was subjected to column IEF in a 5 to 8 pH gradient. The pH of the fractions was tested to establish the gradient (●), and after dialysis to eliminate sucrose and ampholines, these fractions were tested for lytic activity (○).

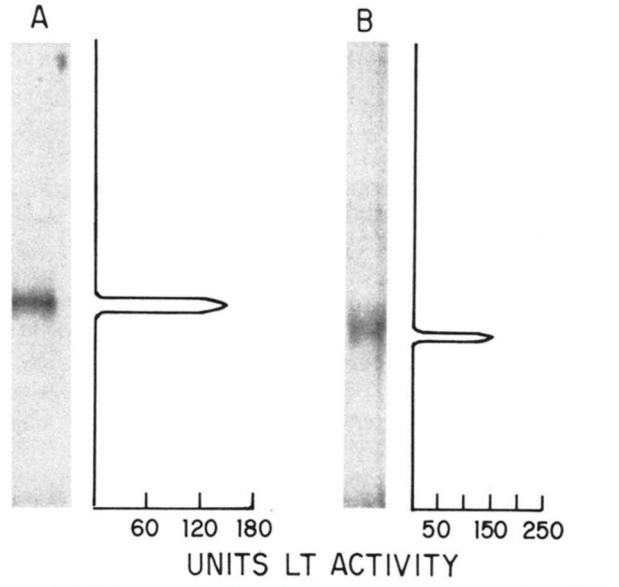

Figure 4. The lytic peaks from the IEF column pH 5.6 to 6 (LT-2) and pH 5 to 6 were pooled, were concentrated, and were subjected to 7% PAGE tube gels. The gels were sliced, material in each slice was eluted, and eluate from each slice was tested for bioactivity. The eluted activity of LT-3 (*A*) and LT-2 (*B*) was rerun on 7% native PAGE slab gels; the gels were cut in half along the axis of migration; one half was silver stained and the other half was cut into 1-mm slices; and the slices were eluted and eluates were tested for bioactivity.

m.w., with contaminant m.w. markers appearing as minor bands below the major LT protein band. However, the LT-2 (Fig. 6*B*) appears as two major protein bands, one at 42,000 m.w. and a second at 21,000 m.w. Additional studies of the LT-2 revealed that this material could be completely dissociated in SDS reducing gels into the smaller 21,000 m.w. form. The two minor protein bands in the LT-2 preparations were not seen on all gels.

In vitro cell lytic ability of purified LT-2 and -3 forms. Target cells were established in microplates and were exposed to various levels of crude supernatant or purified LT-2 and LT-3. Cultures were visually examined every 24 hr up to 72 hr, and the number of viable cells was determined as described in *Materials and Methods.* The results of a single experiment are shown in Table II. The LT-3 form lysed all continuous target cells tested at levels

TABLE I

Purification of LT-2 and -3 forms from PMA-stimulated YM 1.2 cells

Sample	Total Protein	Total Units	Specific Activity (U/mg)
Concentrate: (65 ml)	71 mg	2.4×10^6	3.38×10^4
DEAE fraction: (20 ml)	5.7 mg	6.3×10^5	1.10×10^5
IEF fraction:			
LT-2	16 μg	2.2×10^5	2.64×10^6
LT-3	16 μg	1.1×10^5	1.38×10^6
PAGE:			
LT-2	8 μg	1.0×10^5	1.30×10^7
LT-3	5 μg	0.5×10^5	1.00×10^7

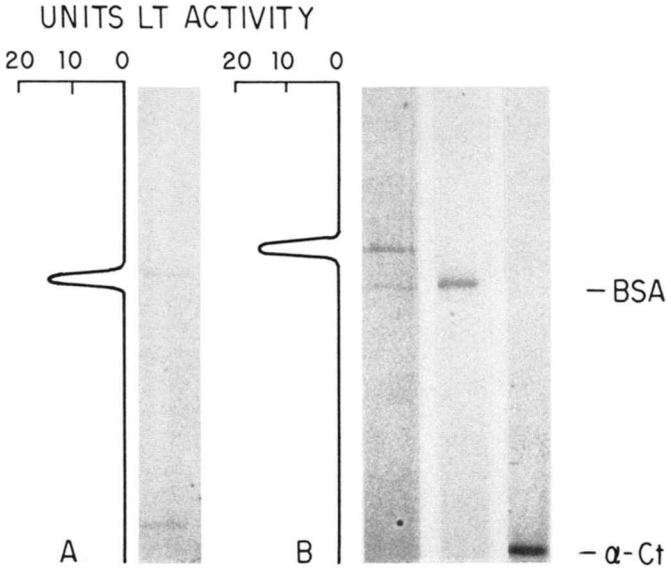

Figure 5. The LT-3 (*A*) and LT-2 (*B*) eluted from 7% native PAGE tube gels were subjected to SDS nonreducing slab gels; the gels were cut in half along the axis of migration; one half was silver stained and the other half was cut into 1-mm slices; and the slices were eluted, were dialyzed, and were tested for bioactivity. Molecular weight markers used were BSA (68,000 m.w.) and α-chymotrypsin (α-Ct 23,000 m.w.).

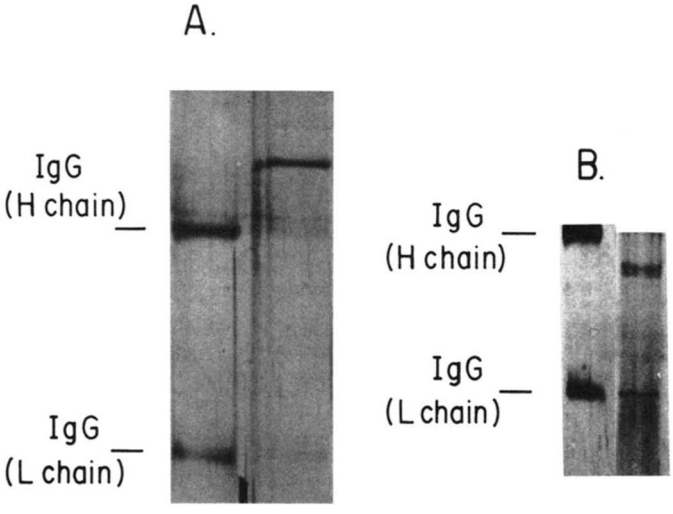

Figure 6. The LT-3 (*A*) and LT-2 (*B*) eluted from 7% native PAGE tube gels were subjected to SDS reducing (0.1% mercaptoethanol) slab gels and were silver stained. Molecular weight markers used were human IgG heavy chain (H chain 50,000 m.w.) and light chain (L chain 23,000 m.w.).

of 10 to 100 U. The LT-2 form was effective on many of the target cells; however, much more material was required (700 to 900 U) to lyse Raji, breast, and colon carcinoma cells. The LT-2 form gave a lytic pattern on K-562, Molt-4F, and Raji cell lines similar to that reported for NK-CF. Unseparated supernatant was as effective as the LT-3 form and lysed all continuous targets except primary fibroblasts (2000 U was the highest level tested). All materials caused lysis of L-929 and HeLa cells within the first 24 hr; however, the other target cells required an additional 48 hr before they were affected.

Capacity of anti-LT and anti-TNF to neutralize LT-2 and -3 in vitro. The results shown in Table III indicate 5 μl of monoclonal anti-TNF completely neutralized the in vitro cell lytic activity of 5 U of rTNF; however, this reagent does not affect the lytic activity of the same amount of α-LT. Five microliters of rabbit anti-LT serum completely neutralized the lytic activity of 5 U of α-LT,

TABLE II

The effect of supernatant and purified LT-2 and -3 from PMA-stimulated YM 1.2 cells on various target cells in vitro[a]

Target Cell	Units of LT causing 50% lysis:		
	Supernatant	LT-2	LT-3
Continuous human:			
HeLa (cervical carcinoma)	10 ± 5	20 ± 5	10 ± 3
Melanoma	35 ± 10	30 ± 7	20 ± 7
Breast carcinoma	42 ± 8	700 ± 30	20 ± 7
Colon carcinoma	80 ± 20	800 ± 25	70 ± 10
K-562 (erythroid leukemia)	100 ± 10	175 ± 10	95 ± 10
Raji (B cell lymphoma)	100 ± 10	900 ± 50	80 ± 9
Molt 4 (T cell lymphoma)	95 ± 10	80 ± 5	15 ± 3
Continuous murine:			
Meth A fibrosarcoma	300	NT[c]	NT
B-16 melanoma	13	NT	NT
Rift fibrosarcoma	12	NT	NT
Nontransformed human:			
WI-38 fibroblasts	NE[b]	NT	NT
GM3468 fibroblasts	NE	NT	NT
Nontransformed murine:			
3T3 Fibroblasts	NE	NT	NT
Primary Fetal Cells	NE	NT	NT

[a] Cells (10,000/well) were established in microplates, then exposed to various dilutions of sample containing 0 to 2000 U of LT activity. After incubation for 72 hr, the number of viable cells remaining was determined as described in *Materials and Methods*. The number of LT units causing a 50% reduction in cell number is expressed. These are the average of two separate studies and four individual microplate wells.
[b] NE, no effect with 2000 U.
[c] NT, not tested.

TABLE III

Neutralization of LT and rTNF by anti-LT, anti-TNF, and a mixture of both reagents in vitro[a]

Antibody	Amount (μl)	% Neutralization of:			
		LT-2[b]	LT-3[b]	α-LT[c]	rTNF
Rabbit anti-LT	1.0	10	0	20	0
	2.5	20	2	79	3
	5.0	34	3	101	2
	7.5	32	5	100	0
	10.0	32	6	101	0
Monoclonal anti-TNF	1.0	4	17	0	13
	2.5	13	52	0	94
	5.0	10	54	7	100
	7.5	14	53	6	98
	10.0	12	52	8	104
Rabbit anti-LT + monoclonal anti-TNF (μl of each)	1.0	89	90	ND[d]	ND
	2.5	93	95	ND	ND
	5.0	100	106	ND	ND

[a] Five units of LT or rTNF were incubated with various amounts of antibody for 30 min at 37°C and were then assayed on L-929 cells for remaining lytic activity. Percent neutralization was calculated as described in *Materials and Methods*.
[b] Fractions from IEF.
[c] Purified α-LT from IR 3.4 cells.
[d] ND, not done.

but had no effect on 5 U of TNF lytic activity. In contrast, 10 μl of anti-LT or anti-TNF alone only partially affected IEF fractions containing LT-2 and -3 activity. However, the combination of 5 μl of each antibody together neutralized 100% of the cell lytic activity in these fractions. Although not shown, we found identical results when we tested two different polyclonal rabbit antisera which neutralized human TNF in vitro (sera described in *Materials and Methods*).

Capacity of rabbit anti-LT and anti-TNF to immunoprecipitate the IEF fraction of LT-2 and LT-3. The IEF fractions of LT-2 and LT-3 were subjected to immunoprecipitation with a monospecific rabbit antibody made against either rTNF or purified α-LT isolated from the continuous human B cell line IR 3.4. The results shown in Table III indicate that antibodies directed at TNF or α-

LT will only partially neutralize the lytic activity of either LT-2 or LT-3; however, the lytic activity of each LT form was almost completely neutralized when both antisera were added together. If these LT forms are a mixture, then immunoprecipitation with a single antibody should only remove one form; however, if both determinants are expressed on one molecule, then immunoprecipitation will remove all of the lytic activity. In these studies, we employed levels of first antibody that gave maximal neutralization of lytic activity and optimal levels of sheep anti-rabbit to achieve complete precipitation of the antigen-antibody complex. The data shown in Table IV-A indicate that immunoprecipitation of LT-2 with anti-LT did not remove a significant amount of lytic activity; however, when the nonprecipitated lytic activity was then tested with monospecific rabbit anti-TNF, a significant amount of activity was neutralized as shown in Table IV-B. The opposite results were observed when the two antisera were tested in reverse order with LT-2 as shown in Table IV-A and IV-B. Thus, this particular IEF LT-2 fraction contains an "α-like" and a "TNF-like" molecule because the removal of one of the LT-2 forms by one antibody was followed by almost complete neutralization by the opposite antibody. This was verified by subjecting the LT-2 to further separation on native PAGE tube gels. Native PAGE resolved these materials into two peaks; one peak which was neutralized by anti-LT but not with anti-TNF, and a second peak which was neutralized by anti-TNF but not with anti-LT. Thus, some α-like material focuses in the pH 5.6 to 6 range, and these two cell lytic forms in this IEF fraction are difficult to resolve. The nonprecipitated lytic activity of the phosphate-buffered saline (PBS)- or normal rabbit serum (NRS)-treated LT-2 gave similar results, as shown in Table III, when these samples were treated with either antibody alone or in combination. Further immunoprecipitation of the nonprecipitated lytic activity with NRS and second antibody failed to remove additional lytic activity.

The same immunoprecipitation procedure was performed on the IEF fraction of LT-3. The results shown in Table V indicate that a majority of the lytic activity was removed with the monospecific rabbit anti-TNF and second antibody, but was not affected by PBS, NRS, or anti-LT and second antibody. Although the data are not shown, the nonprecipitated lytic activity from the PBS, NRS, and anti-LT immunoprecipitation was not affected by anti-LT, was partially neutralized by anti-TNF, and was almost completely neutralized by a mixture of anti-LT and anti-TNF. These results were similar to those shown in Table III.

TABLE IV

Removal of LT-2 in vitro lytic activity by immunoprecipitation with rabbit anti-α-LT or anti-TNF followed by sheep anti-rabbit serum

A.	% Neutralization of LT-2 Activity after Treatment with the Indicated Reagent, Followed by Sheep Anti-rabbit Serum			
	1. PBS	2. NRS	3. Anti-LT	4. Anti-TNF
	0 ± 2[a]	3 ± 2	26 ± 3	11 ± 2
B. Antibody	% Neutralization of nonprecipitated LT-2 activity			
	1. PBS	2. NRS	3. Anti-LT	4. Anti-TNF
Anti-LT	20 ± 5[b]	21 ± 3	8 ± 4	92 ± 5
Anti-TNF	7 ± 1	12 ± 3	90 ± 4	11 ± 1
Anti-LT + anti-TNF	85 ± 5	90 ± 5	94 ± 4	95 ± 4

[a] Fifty units of LT-2 were incubated with 25 μl of PBS, NRS, anti-LT, or anti-TNF for 30 min at 37°C; 25 μl of sheep anti-rabbit serum were then added and were incubated for an additional 30 min at 37°C. The precipitate was removed by centrifugation at 12,500 × G for 5 min, and percent neutralization was calculated as described in *Materials and Methods*.

[b] Five units of nonprecipitated LT-2 activity was mixed with either 5 μl of anti-LT or anti-TNF and 5 μl of NRS or a mixture of 5 μl of each anti-LT and anti-TNF. The supernatants were then tested in the microplate assay on L-929 cells for LT activity, and the percent neutralization was calculated as described in *Materials and Methods*.

TABLE V

Percent neutralization of LT-3 in vitro lytic activity after treatment with NRS or antibody followed by sheep anti-rabbit serum

1. PBS	2. NRS	3. Anti-LT	4. Anti-TNF
0 ± 2[a]	3 ± 1	3 ± 2	80 ± 4

[a] Same as footnote *a* in Table IV.

DISCUSSION

The objective of these studies was to identify a continuous human T cell line releasing toxins similar to those released by primary normal human CTL in quantities that would be sufficient to permit biochemical and functional studies. Only human T cell lines were screened, because previous studies had shown that human B cell lines released mostly α-LT (25). A clone of HUT-102, termed YM 1.2, was developed which spontaneously releases α-LT but which can be induced to release other LT forms when stimulated with PMA in vitro. This pattern of response was similar to that we had observed previously in studies with IL 2-dependent human CTL lines stimulated with lectin or contact with target cells (11). We found that supernatants from PMA-stimulated YM 1.2 cells were lytic for both NK-sensitive and NK-resistant target cells in vitro, and that the cell lytic activity was not affected by anti-LT serum. The LT-2 and LT-3 isolated from these supernatants appears to be biochemically, functionally, and immunologically related to the LT forms released by stimulated human CTL and NK effector cells in vitro. Forms from these cells elute from molecular sieving columns as 65,000 to75,000 m.w. and exhibit an Rf of 0.38 to 0.42 in native PAGE. These forms lyse NK-sensitive and -resistant cells in vitro. As shown in the accompanying companion paper (11), anti-LT or anti-TNF alone only partially neutralized in vitro lytic activity; however, all activity was completely neutralized when the two reagents were mixed. A surprising finding was that LT forms from NK effectors were totally neutralized by the mixture of anti-LT and anti-TNF. These data support the findings of Sverdersy et al. (26), who found that the in vitro lytic activity of NK-CF forms from human NK cells was neutralized with anti-TNF antiserum. Collectively, these data suggest that all lytic forms in supernatants released by antigen- or lectin-stimulated specific and nonspecific effector cells are related to LT and TNF. However, these data also demonstrate that antibody neutralization alone cannot distinguish between certain LT and TNF forms.

Extensive biochemical studies revealed that supernatants from YM 1.2 cells contain three lytically active components. These materials were resolved into three peaks at pH 5 to 5.4, 5.6 to 6, and 6.3 in column IEF. Further biochemical and immunologic studies demonstrated that the material in the 6.3 peak is α-LT, and in certain supernatant lots, some α-LT focused at 5.8. How-

ever, the materials at pH 5 to 5.4 and pH 5.6 to 6 are distinguishable from α-LT. The pH 5.6 to 6 and pH 5 to 5.4 materials were termed LT-2 and LT-3, respectively. After many different biochemical regimens were tested, a final biochemical scheme of DEAE, IEF, and PAGE gels was used which resulted in purification of the LT-2 and LT-3 forms to homogeneity. Purified LT-3 appears as a single band of silver-stained material with an Rf of 0.41 in native PAGE which is coincident with bioactivity. The same material also shows a single stained band of 69,000 m.w. which is coincident with bioactivity in SDS nonreducing gels. Purified LT-2 has a coincident band of staining and biologic activity that co-migrate at Rf 0.38 in native PAGE, and staining and bioactivity that co-migrate at 79,000 m.w. in SDS nonreducing gels. Further studies revealed that the LT-2 can be dissociated into 21,000 m.w. subunits in SDS reducing gels, whereas the LT-3 is unaffected by the same system. The specific activity of each purified LT form, when tested on L-929 cells in vitro is similar and approximately 10^7 U/mg protein. Yields of purified material vary from 1 to 5% of the total starting activity.

The various LT forms in YM 1.2 supernatants each lyse different cells when tested on a panel of continuous and primary target cells in vitro. Although the results are not shown, α-LT is the least effective and only lyses certain continuous cell lines. In contrast, LT-2 and LT-3 were lytic for all of the continuous target cells we employed in these in vitro studies. However, these forms, in a supernatant, were not lytic on primary human fibroblasts derived from different tissue sources. On certain targets, both LT-2 and -3 were equivalent; however, on several targets, a unit of LT-3 was from four to 10 times more effective than LT-2. The lytic capacity of LT-2 appears similar to NK-LT when tested on NK-sensitive (K-562 and Molt-4F cells) and -resistant (Raji) cells, whereas the LT-3 lysed each of these targets. Although the data are not shown, we have just completed studies which demonstrate that interferon is not involved in LT-2- and -3-induced cell lysis in vitro. Rabbit anti-human interferon-α, -β, and -γ does not affect the in vitro lytic activity of these molecules, and interferon activity was not detectable in the unfractionated YM 1.2 supernatant. It is important that interferon is not in these preparations, because both LT and TNF can synergize with interferon. Finally, there were two patterns to cell lysis; the lysis of L-929 and HeLa cells which occurred in 16 to 24 hr, and the lysis of all other target cells which required 48 to 72 hr.

Immunologic and biochemical evidence suggests that the LT-2 form from the YM 1.2 is similar but not identical to TNF released by macrophages and macrophage-like cell lines. The LT-2 molecule expresses antigens in common with TNF and is composed of small m.w. subunits (27). Finally, in studies not shown here, we found that rTNF focuses in the same region as LT-2, pH 5.6 to 6 in our IEF columns. However, the LT-2 subunit is slightly larger (20,000 m.w.) and has a better lytic capacity for continuous human cells in vitro than rTNF (28). Thus, lymphocytes can be a source of TNF-like proteins previously considered to originate from macrophages. These observations support previous reports indicating that certain LT and TNF were immunologically related (2, 29).

The LT-3 molecule appears to be an "LT-TNF-like" form. This material expresses TNF but not LT antigens in aqueous solution; however, LT antigens are present but apparently masked, because they are expressed under other conditions. The LT antigens are expressed during the in vitro cell lytic reaction because anti-LT serum is required to neutralize all of the LT-3 lytic activity on L-929 cells. In studies not shown here, we found that animals immunized with purified LT-3 produced antibodies that would neutralize α-LT in vitro. Several observations support the concept that TNF and LT antigens coexist on the same protein because: 1) almost all LT-3 activity was immunoprecipitated with anti-TNF antibody and second antibody; 2) LT-3 preparations contain a single 69,000 m.w. peptide that does not dissociate into smaller subunits; and 3) LT-3 has a more acidic isoelectric point than either TNF or LT. Thus, LT-3 is either a composite of TNF and LT peptides or a single peptide that shares immunologic determinants with both these molecules. Although data are not shown here, we have found that the LT-3 form does not appear to be glycosylated, and a mixture of recombinant LT and TNF does not express the cell lytic capacity of the LT-3 molecule in vitro. Clearly, a comparison of peptide maps and AA sequence will be required to clarify the relationship of these molecules.

In previous reports in this series, we found that LT forms from lectin-stimulated unseparated human lymphocytes were physically, immunologically, and functionally heterogeneous (1, 15, 30). Further study led to the concept that different lymphocyte subpopulations may be capable of releasing LT molecules with different functional ability, and the NK-LT form was discovered (1, 31–34). The present studies demonstrate that human cytotoxic lymphocytes can release α-LT and "TNF-like" forms in response to different stimuli. These "TNF-like" molecules may be similar to the LT forms previously identified in supernatants from unseparated lymphocytes, termed β-LT (1). Each of the LT-TNF forms appears to lyse a different spectrum of target cells in vitro. Thus, lymphocytes and macrophages have a panel of effector molecules that they can release which may help these effector cells to deal with different types of target cells. However, it is now clear that these molecules have other biologic activities besides lysis and inhibition of cell growth (35). Thus, individual LT-TNF molecules may have different effector functions in vitro and in vivo. With the understanding of the various forms released, the cells that release them, and availability of purified LT and TNF forms for functional studies, their roles in cell-mediated immune reactions may now be clarified.

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