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Authors

Yamamoto, RS
Ware, CF
Granger, GA

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THE HUMAN LT SYSTEM

XI. Identification of LT and "TNF-like" LT Forms from Stimulated Natural Killers, Specific and Nonspecific Cytotoxic Human T Cells in Vitro[1]

ROBERT S. YAMAMOTO,* CARL F. WARE,† AND GALE A. GRANGER[2]*

*From the *Department of Molecular Biology and Biochemistry, University of California, Irvine, CA 92717, and the †Division of Biomedical Sciences, University of California, Riverside, CA 92521*

These studies demonstrate that specific and nonspecific cytolytic human T cells can release LT forms in vitro. Nonspecific cytolytic T cells were derived from IL 2-dependent cultures initiated by allogeneic mixed lymphocyte reaction (AMLR). Specific cytolytic T cells (CTL) were derived from IL 2-dependent T cell clones, initiated by mixed lymphocyte reaction and specific for class II antigens. α-LT was the major lytic component released by these cells in IL 2-dependent cultures. However, on Con A stimulation or contact with target cells, both AMLR and CTL effectors release a new LT form. The new LT form released by AMLR and CTL effectors appear similar, for they both elute from gel filtration at 60,000 to 70,000 m.w. and migrate as a single peak with an Rf of 0.4 on 7% native PAGE tube gels. Moreover, testing these materials on a panel of target cells in vitro indicates that they are both nonspecific, and lyses NK-resistant target cells in vitro. Additional studies revealed that in vitro lytic activity of this form(s) is not affected by either anti-α-LT serum or a monoclonal reagent which inactivates macrophage cell toxins (MCT) and tumor necrosis factor (TNF). However, when these two immunologic reagents are tested together, activity is totally neutralized. Thus, this LT form expresses antigens in common with α-LT, MCT, and TNF. Finally, studies with NK-CF and NK-LT forms revealed that they were also completely neutralized with a mixture of anti-LT and monoclonal anti-TNF antibody. These data suggest that certain macrophage- and lymphocyte-derived cell toxins are interrelated.

Human lymphotoxins (LT)[3] are an inducible family of cell-lytic and growth inhibitory proteins distinguishable from human interferons, which are released by stimulated lymphocytes in vitro (1). Recent data indicate that different types of human lymphocytes can be stimulated in vitro to release similar and distinct LT forms (1–4). Both human T and B lymphocytes and certain continuous human lymphoblastoid cells can be stimulated to release an LT form, termed α, that is 70,000 to 90,000 m.w. (4–6). This material has been shown to exist in two molecular forms, a polymer of 20,000 to 25,000 m.w. peptides and a dimer of a 70,000 m.w. and 20,000 to 25,000 m.w. peptide (6, 7). The small peptide has been sequenced and cloned, and appears to be a component in both α-LT forms (8). Human natural killer (NK) cells stimulated with lectins or contact with target cells release LT forms, termed NK-LT or NK-CF (9, 10). These forms are functionally different from other LT forms because they appear to be species-specific, bind and lyse NK-sensitive target cells, but do not lyse NK-resistant cells in vitro (9, 10). Our previous studies had shown that human T cells could release LT forms that appeared to be distinct from both α-LT and NK-CF forms (4). We decided to examine the capacity of human cytolytic T cells (CTL) to release LT forms in vitro.

Both antigen-specific and -nonspecific human CTL can be generated by in vitro techniques. Co-culture of human peripheral blood lymphocytes (PBL) with allogeneic continuous B cell lines, termed the allogeneic mixed lymphocyte reaction (AMLR), generates both specific and nonspecific cytolytic T cells (11–13). Nonspecific AMLR effector cells are $OKT4^+$ and $OKT8^+$, and have the ability to lyse a broad range of target cells, including NK-sensitive and -resistance target cells (14). Specific cytotoxic T killers directed at class I or II antigens can be initiated by mixed lymphocyte reaction, specific effectors cloned and then maintained in culture with interleukin 2 (IL 2) and the specific target cells (15, 16). The present studies identify that human CTL can release multiple LT forms in vitro. One of these, a highly cell lytic form, appears to be related to both lymphocyte-derived α-LT and macrophage cell toxins (MCT) and tumor necrosis factors (TNF).

MATERIALS AND METHODS

Peripheral blood mononuclear cells (PBM). Human mononuclear cells were obtained from peripheral blood of normal donors by the Ficoll-Hypaque technique of Boyum (17). Fifty to 100 milliliters of peripheral blood were collected, and after separation on Ficoll-Hypaque gradients, the cells were suspended in serum-free RPMI 1640 (GIBCO, Grand Island, NY) (RPMI-0%). These cells were 99 to 100% viable, as determined by Eosin Y staining.

Target cells. We employed L-929 (continuous murine fibroblasts), HeLa (human carcinoma of the cervix), K-562 (human erythroid leukemia), MOLT-4F (human T cell leukemia), and three continuous

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[1] This research was supported by Rheumatic Diseases Research Foundation Grant 1882 and Grant No. 9-440850-29169 from the National Institutes of Health.
[2] To whom correspondence should be addressed.
[3] Abbreviations used in this paper: LT, lymphotoxins; MCT, macrophage cell toxins; TNF, tumor necrosis factors; CTL, cytolytic T cells; AMLR, allogeneic mixed lymphocyte reaction; PBL, peripheral blood lymphocytes; PBM, peripheral blood mononuclear cells; E:T, effector to target; rTNF, recombinant human TNF.

human B cell lines (Raji, RPMI-1788, and WI-L2) as target cells. The L-929 and HeLa cells were maintained as monolayer cultures in 32-oz prescription bottles in an atmosphere of 95% air, 5% CO_2, and were passed biweekly. Suspension cultures of K-562, MOLT-4F, Raji, RPMI-1788, and WI-L2 were grown in Corning T-25-cm^2 tissue culture flasks in an atmosphere of 95% air, 5% CO_2, and were passed biweekly. The L-929 cells were maintained in RPMI 1640 plus 3% heat-inactivated fetal calf serum (FCS; GIBCO) (RPMI-3%), and human cells were maintained in RPMI-10%.

Lymphocyte culture and supernatant production: activated NK cells. The NK cells were preactivated by co-culturing PBM in FCS as described (9). The NK cultures were established at a density of 2×10^6 cells/ml in RPMI-10% and were incubated at 37°C for 5 days. After the 5-day incubation in RPMI-10%, the NK were collected by centrifugation at 300 × G for 5 min. These cells were either used for direct cell cytolysis or were stimulated with 20 μg/ml concanavalin A (Con A) for 5 hr at 37°C at a density of 10^6 cells/ml in RPMI-10%. The supernatants were collected by centrifugation at 500 × G for 10 min and were either used immediately or were frozen at −70°C.

Allogeneic mixed lymphocyte reaction (AMLR). The AMLR effectors were generated by co-culturing PBL with mitomycin C-treated continuous B cell lines, without feeder cells, at a ratio of 10:1 effector to target (E:T) cells at a density of 2×10^6 cells/ml in RPMI-10% as described (11–13). After 5 days at 37°C, the effector cells in these cultures were collected by centrifugation at 300 × G for 5 min. Lymphocytes were restimulated at 5-day intervals by co-culture with the sensitizing cell line at a density of 10^6 cells/ml at an E:T ratio of 5 to 1 plus 50% IL 2-containing medium (Bethesda Research Laboratories, Gaithersburg, MD). Supernatants were generated by stimulation of these cells at a density of 10^6 cells/ml in RPMI-10% with 10 μg/ml Con A. After 5 hr at 37°C, the cells were removed by centrifugation at 500 × G for 10 min, and supernatants were either used or were frozen at −70°C.

CTL lines. Human PBL were co-cultured with Daudi cells as described by Krensky et al. (18). Clones of CTL were generated by the limiting dilution technique of Levey et al. (19). In brief, cell suspensions were diluted and dispensed so that one cell was delivered per two microplate wells. The initial cultures were maintained on a feeder monolayer of 5×10^3 BALB/c peritoneal macrophages per well; however, once established, the feeder layers were eliminated. Lines were maintained by passage and restimulation every 3 days with irradiated Daudi cells at a ratio of 5:1 E:T cell in the presence of 50% human IL 2 (Bethesda Research Laboratories) and fresh RPMI-10%. In several cases, a clone was subcloned before we employed them in our studies. Each clone was tested with the following monoclonal reagents before use: OKT-3, OKT-4, and OKT-8 (Ortho Pharmaceutical Corp., Raritan, NJ). Three clones that induced specific lysis of Daudi cells were selected. These cells were 99% $OKT3^+$, 97% $OKT4^+$ and $OKT8^-$. Cells from clone I were employed for a majority of these studies; 5×10^5 were stimulated with 20 μg/ml Con A for 5 hr, the cells were removed, and supernatant was tested or stored as described above. Supernatants from the THP-1-0 human macrophage cell line, containing MCT and the IR 3.4 lymphoblastoid cell line, were generated as described (5, 20).

^{51}Cr-release assay. Two types of assays were conducted, one with effector and target cell mixtures and one with target cells incubated with supernatants from effector cells. All assays were conducted in round-bottomed microcytotoxicity plates (Flow Laboratories, McLean, VA). One milliliter of target cells (10^7 cells/ml) in RPMI-0% was labeled with 100 μCi of ^{51}Cr (305 mCi/1 mg; New England Nuclear, Boston, MA). After 1 to 2 hr at 37°C, the cells were washed three times (300 × G for 5 min) with cold RPMI-10%. Labeled target cells (2×10^4 in 0.02 ml) were added to microtiter wells with various numbers of effector cells or different amounts of supernatant. The plates were then incubated at 37°C for 3 hr for direct cell lysis and 12 hr for supernatant lysis. The release of ^{51}Cr label was measured with the Titertek Supernatant Collection System (Flow Laboratories) and was quantitated in an automated gamma counter (Beckman, Fullerton, CA). The total ^{51}Cr releasable (90 to 95% of total counts) was determined by lysing the cells with 0.1 ml of 3% (w/v) sodium dodecyl sulfate solution. Spontaneous ^{51}Cr release was the same (1 to 2%/hr) from the target cells when incubated in RPMI 1640 medium containing lectin, or lectin free. Percent cell lysis was determined by the following formula:

$$\frac{\text{Experimental release} - \text{spontaneous release}}{\text{Total release} - \text{spontaneous release}} \times 100 = \%\ \text{lysis}$$

LT assay. Two types of LT microassays were employed, one which determines the amount of activity present in a sample, and a second which only indicates its presence or absence. Both assays have been described previously in detail (2, 21). Monolayers of L-929 or HeLa cells were established in flat-bottomed microtiter plates at a density of 15,000 cells/well in RPMI-3% or RPMI-10%, respectively, containing 0.5 μg/ml mitomycin C. After 24 hr at 37°C, serial dilutions of LT-containing sample or control sample in a fixed volume of 25 μl were added to triplicate wells. After 24 hr at 37°C, the cells were stained with crystal violet, solubilized, and the amount of stain in each well was quantitated in a Titertek Multiskan Plate Reader (Flow Laboratories) equipped with a 580-nm Multiskan Interference Filter. The number of cells in the well is directly proportional to the amount of absorbance. A unit of LT in a supernatant or sample is expressed as the reciprocal of the dilution affecting a 50% reduction in cells (22).

Biochemical techniques. The methods employed for fractionation of LT activity have been described (2).

Molecular sieving. Degassed Ultrogel AcA 44 (LKB, Upsala, Sweden) was poured to a bed height of 95 to 100 cm in a 2.5 x 120-cm siliclad-coated column equilibrated in 10 mM potassium phosphate, 0.1 mM EDTA, at pH 7.2. The column was calibrated numerous times over the period of these studies with blue dextran (2×10^6 m.w.), human IgG (150,000 m.w.), hemoglobin (64,000 m.w.), α-chymotrypsin (23,000 m.w.), and phenol red (395 m.w.). Six-milliliter fractions were collected at a flow rate of 24 ml/hr. Samples of 1 to 1.5 ml were chromatographed on this column, and 5 to 10 μl from each fraction were assayed for LT activity.

DEAE cellulose chromatography. In brief, DEAE (Whatman, Maidstone, UK) was poured to a bed height of 20 cm in a 2.5 × 34-cm siliclad-coated glass column that was equilibrated in 10 mM Tris-HCl, pH 8, 0.1 mM EDTA, and 0.05 M NaCl (low-salt Tris buffer). Supernatants or samples were dialyzed against 1000 vol of low-salt Tris buffer for 24 hr at 4°C. Protein was eluted with a linear 120- to 300-ml gradient from 0.05 to 0.3 M NaCl in low-salt Tris buffer followed by 50 ml of 1 M NaCl in the same buffer. Six-milliliter fractions were collected at a flow rate of 30 ml/hr and were tested for LT activity.

Polyacrylamide disc gel electrophoresis (PAGE). A 100- to 200-μl sample in 20% sucrose was applied to a 0.5 × 8-cm gel consisting of a 1-cm 3% acrylamide stacking gel and a 7-cm 7% acrylamide separating gel in 50 mM Tris-glycine, pH 9.6. The sample was subjected to electrophoresis at 4 mA/gel at 4°C. The gels were then cut into 2-mm slices, and each slice was incubated in 0.3 ml RPMI-3% for 24 hr at 4°C. A 10-μl sample of the eluate was then tested in triplicate on L-929 cells for LT activity. The Rf values were calculated with reference to the migration of a bromphenol blue marker.

Antisera and neutralization of cell lytic activity in vitro. Generation of rabbit antiserum against purified α-LT from a continuous human lymphoblastoid cell line, IR 3.4, and from normal human lymphocytes has been described in detail (23, 24). A murine monoclonal reagent that neutralizes the in vitro lytic activity of human TNF, but not human α-LT, was obtained from Biogen Corp. (Boston, MA). This monoclonal reagent was derived from animals that had been immunized with recombinant human TNF (rTNF). In vitro antibody neutralization tests were performed in a standard manner; all active samples were first pretested and standardized so they contained 4 to 5 U of cell lytic activity per milliliter, standardized samples were then incubated with antibody preparations or normal rabbit serum for 30 min at 37°C, and lytic activity was then determined in the microplate assay. 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

Cell lytic capacity of human NK and AMLR effector cells in vitro. Effector lymphocytes generated from the AMLR and NK cultures after various time intervals were assayed for their ability to lyse a panel of target cells in a 3-hr ^{51}Cr-release assay. The effector cells generated from the AMLR cultures exhibited a strong nonspecific cytotoxic response against NK-sensitive and -resistant target cells after 5 and 30 days in culture, as shown in Table I. The only target cells the NK cultures lysed consistently were the NK-sensitive K-562 and MOLT-4F cells. The results of these experiments were generally the same for effectors generated from four different lympho-

TABLE I

Lysis of ^{51}Cr-labeled allogeneic target cells by AMLR and NK effectors in a 3-hr ^{51}Cr-release assay in vitro[a]

Effector Cell	Target Cells	% Specific ^{51}Cr Release at E:T Cell Ratios of	
		25:1	10:1
NK	K-562	42 ± 3	24 ± 2
	MOLT-4F	64 ± 5	49 ± 4
	RAJI	6 ± 1	1 ± 1
	RPMI-1788	11 ± 3	7 ± 1
	HeLa	11 ± 2	6 ± 2
5-day AMLR	K-562	55 ± 2	45 ± 1
	MOLT-4F	45 ± 2	41 ± 1
	RAJI	42 ± 1	38 ± 7
	RPMI-1788	41 ± 0	26 ± 1
	HeLa	26 ± 6	16 ± 4
30-day AMLR	K-562	75 ± 1	60 ± 4
	MOLT-4F	41 ± 1	35 ± 9
	RAJI	67 ± 9	41 ± 4
	RPMI-1788	36 ± 3	19 ± 4
	HeLa	25 ± 4	17 ± 3

[a] Human AMLR and NK effector cells were generated as described in *Materials and Methods*. Effector cells were mixed with ^{51}Cr-labeled target cells at various ratios and were then added to triplicate wells of a microtiter plate. After 3 hr, the amount of ^{51}Cr released into the supernatant as compared to controls was calculated as described in *Materials and Methods*.

TABLE II

Capacity of supernatants from lectin-stimulated NK and AMLR cells to cause lysis of various allogeneic target cells in vitro[a]

Target cells	% Specific ^{51}Cr Release Induced by Supernatants from		
	NK	5-day AMLR	30-day AMLR
K-562	28 ± 4	22 ± 4	26 ± 3
MOLT-4F	45 ± 2	34 ± 4	30 ± 2
RAJI	4 ± 2	23 ± 3	18 ± 4
RPMI-1788	5 ± 3	34 ± 6	23 ± 2
WI-L2	2 ± 4	20 ± 2	19 ± 2
HeLa	3 ± 2	45 ± 3	53 ± 6

[a] NK and AMLR effectors were stimulated with Con A for 5 hr; supernatants were then collected and were tested for LT activity on ^{51}Cr-labeled target cells in a 12-hr microplate assay as described in *Materials and Methods*. Specific ^{51}Cr release was determined as described for Table I.

cyte donors. We found that NK cultures failed to survive if maintained beyond 5 days in vitro. The AMLR cultures could be maintained for 60 to 90 days with IL 2 and the stimulating B cell line. The AMLR effectors were 70% OKT3.$^+$

Capacity of supernatants from AMLR and NK cells to induce lysis of ^{51}Cr-labeled allogeneic target cells in vitro. Lectin-stimulated supernatants from AMLR and NK cultures were immediately tested for LT activity in a standard LT assay employing L-929 cells, and then indicated levels of LT activity were tested on NK-sensitive and -resistant cells in a 12-hr ^{51}Cr-release assay. The LT activity detected on L-929 cells in these superntants varied from 50 to 200 U/ml. The data from a single experiment are presented in Table II, and indicate that the supernatants from the NK cultures lysed the NK-sensitive K-562 and MOLT-4F target cells but had no effect on the NK-resistant targets. In contrast, supernatants generated from 5- and 30-day AMLR cultures were able to induce lysis of both NK-sensitive and -resistant targets. Additional studies gave similar results; however, NK lysis ranged from 20 to 65% of NK-sensitive target cells, and AMLR lysis ranged from 15 to 75% lysis of both NK-sensitive and -resistant target cells. Human HeLa cells were not lysed by NK effectors but were lysed by both AMLR effectors and their supernatants. Therefore, we employed the HeLa cell as an indicator of the cell lytic LT forms from AMLR cultures. A control supernatant of RPMI-10% + 10 μg of lectin per milliliter had no effect in these assays.

Gel filtration chromatography of AMLR supernatants. Supernatants from Con A-stimulated and nonstimulated AMLR cultures were concentrated and were subjected to gel filtration chromatography on AcA 44 columns as described in *Materials and Methods*. Shown in Figure 1*a* is the cytotoxic elution profile of a 72-hr supernatant from a nonstimulated 30-day AMLR culture as tested on L-929 and HeLa cells. When tested on L-929 cells, the cytolytic activity can be resolved into one major α m.w. LT class (80,000 to 100,000 m.w.) and three minor m.w. classes; however, only two of the four classes have any significant cytolytic activity on HeLa target cells. The nonstimulated supernatants from the 5- and 15-day cultures gave similar cytotoxic profiles, as shown in Figure 1*a*. The data shown in Figure 1*b* indicate that the lectin-stimulated supernatant from these same cells contains cell lytic material that is predominantly 60,000 to 70,000 m.w. Although not shown, a similar profile was seen for 5- and 15-day lectin-stimulated supernatants. This peak of lytic activity was effective on both HeLa and L-929 target cells.

Ion-exchange chromatography and PAGE of AMLR LT from gel filtration. The active fractions from gel fil-

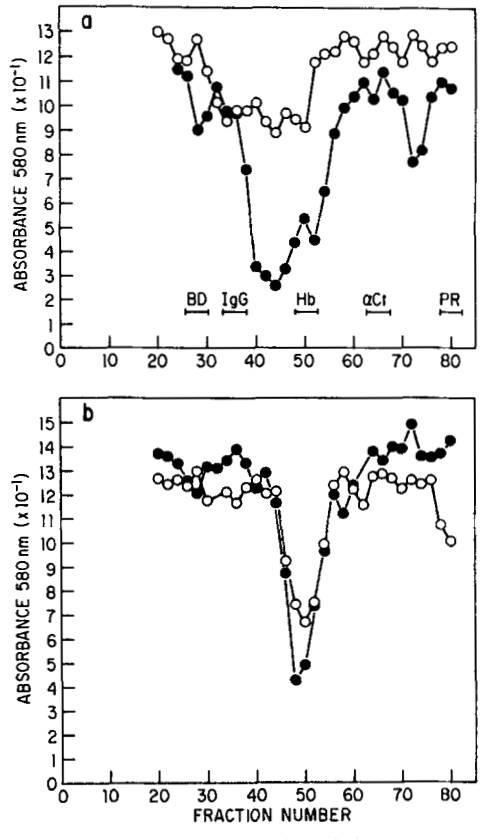

Figure 1. Cytotoxic elution profile of: *a*, 3-day spent medium from nonstimulated AMLR culture; *b*, 30-day AMLR culture stimulated with Con A as described in *Materials and Methods*, on an Ultrogel AcA 44 molecular sieving column. The elution profile of various m.w. markers is indicated by the horizontal bars: blue dextran (BD >200,000 m.w.), human IgG (150,000 m.w.), hemaglobin (Hb 64,000 m.w.), α-chymotrypsin (α-Ct 23,000 m.w.), and phenol red (PR 395 m.w.). ●, Cytotoxic activity on L-929 cells; ○, cytotoxic activity on HeLa cells measured by absorbances as described in *Materials and Methods*.

tration were pooled, were concentrated, were dialyzed against low-salt Tris buffer for 24 hr, and were then chromatographed on a DEAE cellulose column pre-equilibrated in the same buffer. The cytotoxic elution profile of this material is shown in Figure 2. The lytic activity as detected on both L-929 and HeLa target cells elutes off the DEAE column as one cytotoxic peak in the region of 0.075 M NaCl. Although this figure represents an individual elution profile, similar results have been obtained in numerous separations. Fractions from DEAE containing the lytic activity were pooled, were concentrated, and were subjected to 7% PAGE as described in *Materials and Methods.* The results shown in Figure 3 indicate that the lytic activity migrates as a single peak with an Rf of 0.42 when tested on both L-929 and HeLa target cells.

Gel filtration and PAGE of LT forms from CTL clones. Cells, 5×10^5/ml, from clone 1 were stimulated with 20 μg/ml Con A for 5 hr as described in *Materials and Methods.* Five milliliters of the supernatant were concentrated 25 times and were subjected to molecular sieving on a 0.8 x 22-cm Ultrogel AcA 44 column equilibrated in 10 mM potassium phosphate, 0.1 mM EDTA, at pH 7. One hundred microliters were chromatographed and fractions were collected at the flow rate of 4 ml/hr. As shown in Figure 4, there appears to be only one peak of lytic activity that elutes in the m.w. range of 60,000 to 70,000 when assayed on L-929 cells. The fractions containing lytic activity were then pooled and were concentrated 20 times in a 3-ml Amicon Stirred Cell, using a YM 10 membrane. This concentrated sample was subjected to PAGE as described in *Materials and Methods.* As shown in Figure 5, the lytic activity migrates as a single peak with an Rf of 0.39 when tested on L-929 target cells.

Testing supernatants and fractions with anti-LT and anti-TNF serum. Rabbit anti-LT serum and monoclonal anti-rTNF were tested for their ability to neutralize the lytic activity of supernatant and fractionated samples from stimulated and unstimulated AMLR and CTL cultures. These reagents were first tested against purified human α-LT from IR 3.4 lymphoblastoid cells (24) and rTNF. A dose curve using varying amounts of each immunologic reagent tested against a constant level (5 U) of either α-LT or rTNF is shown in Table III. Five microliters

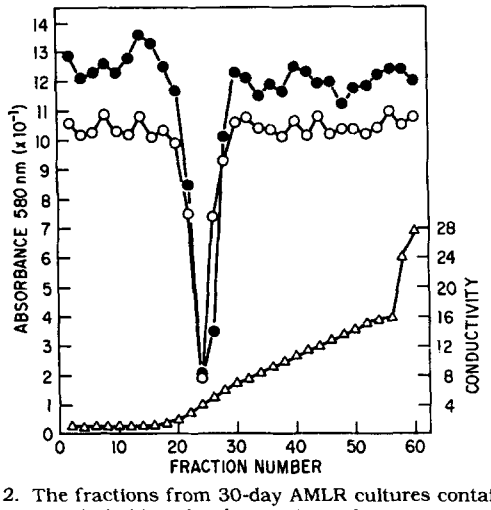

Figure 2. The fractions from 30-day AMLR cultures containing lytic activity from an AcA 44 molecular sieving column were pooled, were concentrated, were dialyzed against low-salt Tris buffer, and were applied to a DEAE column as described in *Materials and Methods.* Fractions from the DEAE column were assayed for conductivity (Δ), and lytic activity was expressed as absorbance as described in *Materials and Methods* on L-929 cells (●) and HeLa cells (○).

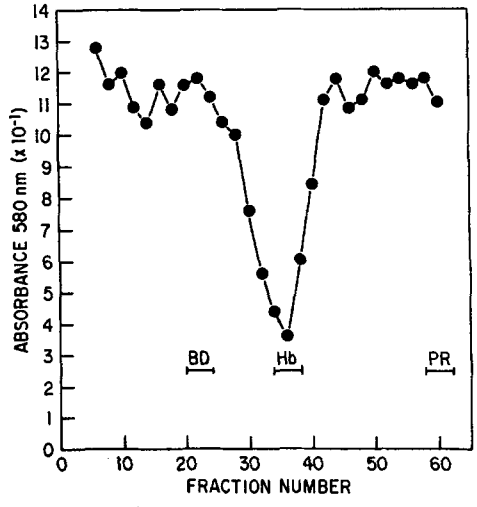

Figure 4. Cytotoxic elution profile as assayed on L-929 cells of a 5-hr lectin-stimulated CTL culture on a 0.8 x 22-cm Ultrogel AcA 44 molecular sieving column. The m.w. markers were blue dextran (BD >200,000 m.w.), hemaglobin (Hb 64,000 m.w.), and phenol red (PR 395 m.w.). Cytotoxicity is expressed as absorbance as described in *Materials and Methods.*

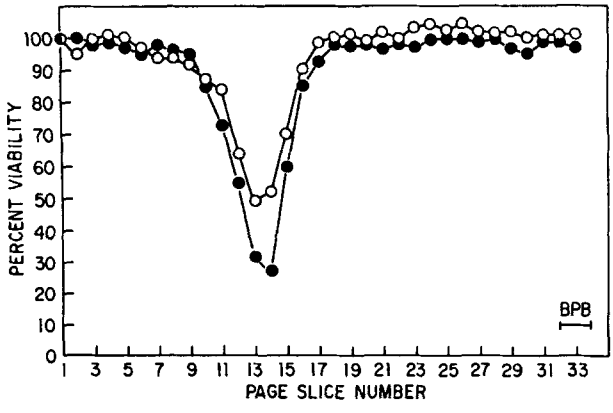

Figure 3. Fractions containing lytic activity from DEAE were concentrated and were applied to a 7% native PAGE tube gel as described in *Materials and Methods.* The PAGE gel was then sliced and bioactivity was eluted with RPMI-3% for 24 hr at 4°C. ●, Cytotoxic activity on L-929 cells; ○, cytotoxic activity on HeLa cells. BPB, Bromphenol blue.

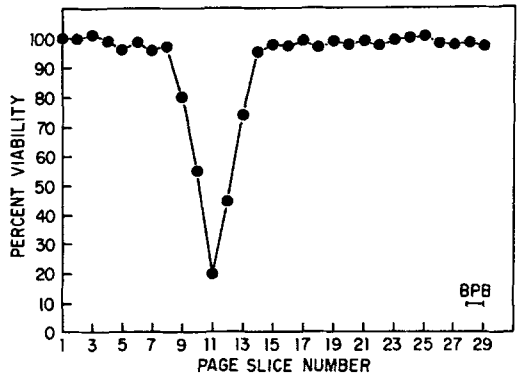

Figure 5. Fractions containing lytic activity from an AcA 44 molecular sieving column were concentrated and were applied to a 7% native PAGE tube gel. The PAGE gel was then sliced, and bioactivity was eluted with RPMI-3% for 24 hr at 4°C and was assayed on L-929 cells. BPB, Bromphenol blue.

TABLE III

The effect of anti-LT and anti-TNF on the lytic activity of LT and rTNF on L929 cells in vitro[a]

Antiserum	Amount Employed (μl)	% Neutralization with	
		α-LT[b]	rTNF
Anti-LT	1.0	20 ± 2	0 ± 2
	2.5	79 ± 6	3 ± 4
	5.0	101 ± 2	2 ± 1
	7.5	100 ± 3	−2 ± 2
	10.0	101 ± 5	−6 ± 3
Anti-rTNF	1.0	−2 ± 4	13 ± 4
	2.5	−5 ± 1	94 ± 6
	5.0	7 ± 4	100 ± 3
	7.5	6 ± 3	98 ± 3
	10.0	8 ± 5	104 ± 2

[a] Prestandardized samples containing 5 U of either α-LT or rTNF were incubated with various doses of antisera for 30 min and were then assayed on L-929 cells for lytic activity. Percent neutralization was calculated, employing normal rabbit and mouse serum as controls, as described in *Materials and Methods*.

[b] Purified α-LT was obtained from the continuous human B cell line IR 3.4.

TABLE IV

Neutralization of lytic activity in supernatants and fractions from AMLR and CTL cultures by rabbit anti-LT sera tested on L929 cells in vitro[a]

Preparation	% Neutralization by	
	anti-LT[b]	anti-LT[c]
AMLR spent medium	96 ± 3	96 ± 2
AMLR lectin-stimulated medium	6 ± 3	7 ± 6
AMLR AcA 44 fraction	2 ± 2	2 ± 1
AMLR DEAE fraction	1 ± 2	2 ± 1
AMLR PAGE fraction	7 ± 2	7 ± 2
CTL spent medium	97 ± 5	98 ± 6
CTL lectin-stimulated medium	5 ± 3	6 ± 3
CTL AcA 44 fraction	1 ± 2	1 ± 1
CTL PAGE fraction	1 ± 1	1 ± 1
α-LT	101 ± 4	100 ± 5
rTNF	5 ± 2	6 ± 3

[a] Samples from each preparation, first adjusted to contain 5 U of activity, were mixed with 5 μl of antiserum or normal rabbit serum and were then added to duplicate microplate wells containing 15,000 L-929 cells per well. Cell viability was assayed after 16 to 20 hr and percent neutralization was calculated by comparison of test and control wells as described in *Materials and Methods*.

[b] Antiserum to purified α-LT from normal human lymphocytes.

[c] Antiserum to purified α-LT from IR 3.4 human lymphoblastoid cells.

of either reagent completely neutralized the lytic activity of their respective antigens but had no effect on the lytic activity of the other effector molecule. The results shown in Table IV indicate that unstimulated supernatants contain mostly α-LT because they were almost completely neutralized with anti-LT serum; however, stimulated supernatants were unaffected by this same serum. It is also clear that the peak of lytic activity observed when stimulated medium from AMLR and CTL cells is subjected to gel filtration, DEAE, and PAGE is also unaffected by anti-LT serum.

Anti-LT and anti-rTNF were tested to determine whether they would affect the lytic activity of supernatants from NK, AMLR, and CTL cells in vitro. First, a dose curve was established to determine the ability of the anti-TNF and anti-LT to neutralize their respective antigens, which is shown in Table III. These immunologic reagents were then tested on supernatant lytic activity obtained from the different effector cell populations. The amount of LT was standardized to 5 U and was tested in the the presence of different amounts of antibody. The immunologic reagents were tested singly or when mixed together. The results shown in Table V indicate that the

TABLE V

The effect of anti-LT and anti-rTNF sera on the in vitro cell lytic activity of supernatants derived from stimulated normal and continuous human lymphoid cells[a]

Supernatant Source	Antiserum Employed		
	anti-LT	anti-rTNF	anti-LT + anti-rTNF
AMLR (Con A-stimulated)	24 ± 3	15 ± 2	97 ± 2
CTL (Con A-stimulated)	15 ± 4	0 ± 0	97 ± 7
NK (Con A-stimulated)	−3 ± 8	25 ± 0	81 ± 0
AMLR (antigen-stimulated)[b]	13 ± 6	10 ± 2	93 ± 4
CTL (antigen-stimulated)[b]	−5 ± 3	15 ± 6	100 ± 0
NK (antigen-stimulated)[b]	2 ± 3	27 ± 5	98 ± 6
rTNF[c]	−2 ± 3	101 ± 2	ND[f]
Purified α-LT (IR 3.4 cells)	102 ± 5	7 ± 3	ND
THP-1-0 cells (PMA-stimulated)[d]	−3 ± 6	98 ± 4	ND
IR 3.4 cells (PMA-stimulated)[e]	100 ± 4	12 ± 3	ND

[a] Five units of supernatant activity from the different cell populations were added to: A) 5 μl of anti-LT or anti-rTNF serum, or B) 5 μl composed of 2.5 μl of anti-LT and 2.5 μl of anti-rTNF. The mixture was tested for lytic activity on L-929 cells in the microplate assay. After 16 hr at 37°C, the percent neutralization of lytic activity was determined as described in *Materials and Methods*.

[b] Supernatant from AMLR, CTL, and NK effectors was obtained from each culture after each effector cell population had been incubated for 5 hr with lymphoblastoid, Daudi, and K-562 targets, respectively, at a 5:1 E:T cell ratio.

[c] Recombinant TNF obtained from the Biogen Corp.

[d] Supernatant generated by 48-hr PMA stimulation of the continuous monocytic cell line THP-1-0 which contains MCT-TNF as described by Armstrong et al. (20).

[e] Supernatant generated by 72-hr PMA stimulation of the continuous B cell line IR 3.4 as described by Yamamoto et al. (5).

[f] ND, not done.

anti-LT serum neutralized all lytic activity in the supernatants from the IR 3.4 lymphoblastoid cells. The monoclonal antibody to rTNF neutralized the lytic activity of the rTNF and the supernatant from the THP-1-0 monocytic cell line. However, neither of these antisera alone neutralized lytic activity in the supernatants from AMLR, CTL, or NK cells stimulated with lectin or contact with target cells. In contrast, the lytic activity expressed in these supernatants was almost totally neutralized when tested against a combination of the two immunologic reagents. The same results were observed with a polyclonal antibody generated against TNF (data not shown).

DISCUSSION

These studies demonstrate that cytolytic human T cells induced by AMLR can release different LT forms in vitro. The cells in these IL 2-dependent cultures can lyse many different target cells in vitro, including NK-sensitive and -resistant targets. The AMLR effector cells release α-LT when proliferating in the presence of IL 2; however, on lectin stimulation or co-culture with target cells, they release another form(s) of LT. This other component(s) is nonspecific and has the ability to lyse a broad range of target cell types in vitro, including NK-resistant and -sensitive target cells. The lytic activity in these supernatants appears to be due to a component(s) which has m.w. of 60,000 to 70,000, elutes from DEAE and PAGE as a single peak, and is not neutralized by anti-α-LT serum. These results indicate that AMLR effector cells can release different LT forms in response to different stimulatory signals. However, this cannot be stated with certainty because AMLR cells are a mixed cell population and the different LT form(s) could originate from separate cell populations.

Cloned cytotoxic human T cells can also release differ-

ent LT forms in vitro. These effector cells were OKT3$^+$, OKT4$^+$, and OKT8$^-$; they had specificity for class II antigens expressed on Daudi cells. We found that, like the AMLR effectors, the CTL release α-LT when proliferating in vitro in the presence of IL 2. They also secrete another LT form(s) when stimulated with lectin or on contact with target cells. The activity in these supernatants also appears to be due to a component(s) that elutes from gel filtration as a 65,000 to 70,000 m.w. and that has an Rf of 0.4 in native PAGE, which is similar to the Rf of material from AMLR cultures. The CTL-derived material is lytic for NK-resistant target cells in vitro and is not neutralized by anti-LT serum. The data are not extensive; however, they are supportive of the possibility that the material(s) in the supernatants from AMLR and CTL effectors may be similar. These studies indicate that cells derived from a single effector T cell can produce different LT forms in response to different stimulating signals. The material in both AMLR and CTL cultures does not appear to be NK-LT or NK-CF because it lyses NK-sensitive and -resistant targets in vitro. Thus, this may be a new LT form, perhaps unique to CTL.

Immunologic studies indicate that the T cell LT form is related to both α-LT and MCT-TNF derived from macrophages. The in vitro lytic activity of the material in the stimulated supernatants from AMLR and CTL cells was not neutralized by either anti-LT or anti-TNF antiserum alone; however, activity was totally inhibited by a mixture of these antisera. These are provocative results, because they indicate that this material is either an assemblage of LT and MCT or an LT form that shares antigenic determinants with both proteins. An equally surprising result was the finding that the same mixture of antisera would also neutralize the lytic activity of supernatants containing NK-LT forms. The major detriment of these studies is that they involve impure preparations and mixtures of effector molecules; however, these findings suggest the new concept that lytic components from both lymphocytes and macophages and from different populations of effector lymphocytes are interrelated. Two previous studies also reported on the immunologic relationship between lymphocyte-derived LT forms and macrophage-derived cytotoxic forms from both mice and guinea pigs (25, 26). Further biochemical and structural studies of these interesting proteins are necessary to clearly distinguish the nature of the relationships between these effector molecules.

Further studies of the LT form(s) in supernatants from these effector cells present several serious problems. Although AMLR cultures are a source of enough supernatant for preliminary biochemical studies, these cultures are really not satisfactory because they are a mixture of cells. In contrast, the CTL are a homogeneous cell population, but there are not enough cells to produce the necessary quantities of supernatant. What is needed is a continuous human T cell line or a T cell hybridoma that produces the active material(s) in quantity for further studies. The accompanying companion paper which follows (27) describes the identification of such a cell line and the purification of two active materials.

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