Title

Identification of human lymphocyte-derived lymphotoxins with binding and cell-lytic activity on NK-sensitive cell lines in vitro

Journal

Cellular Immunology, 77(1)

ISSN

0008-8749

Authors

Weitzen, ML
Yamamoto, RS
Granger, GA

Publication Date

1983-04-01

DOI

10.1016/0008-8749(83)90004-7

CELLULAR IMMUNOLOGY 77, 30–41 (1983)

Identification of Human Lymphocyte-Derived Lymphotoxins with Binding and Cell-Lytic Activity on NK-Sensitive Cell Lines *in Vitro*[1]

M. L. WEITZEN, R. S. YAMAMOTO, AND G. A. GRANGER

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

Received August 17, 1982; accepted January 6, 1983

Supernatants obtained from lectin-restimulated, preactivated, human peripheral blood lymphocytes rapidly released (5–24 hr) high levels of lymphotoxin (LT) activity *in vitro*. Peripheral blood lymphocytes were preactivated by coculturing with either fetal calf serum or with allogeneic continuous B-cell lines (LCCL) which were treated with mitomycin C. These supernatants contained a population of L-929 cell-lytic LT forms which also selectively bind to the NK-sensitive K-562 cell. However, lytic LT forms for L-929 cells from cPBL and LCCL cultures did not bind to the NK-sensitive MOLT-4 or NK-resistant Raji cells. Additional studies reveal these supernatants contain a second set of LT forms which have cell-binding and cell-lytic activity detectable on MOLT-4 and K-562 cells in a 12 to 18 hr ^{51}Cr-release assay. Cell-lytic form(s) for the MOLT-4 and K-562 cells were not stable for more than a week at −20°C. These findings indicate that materials with LT activity are heterogeneous with respect to their capacity to recognize common and discrete cell-surface components on different types of target cells *in vitro*.

INTRODUCTION

Lymphotoxins (LT) are cytotostatic and cytotoxic glycoproteins produced *in vitro* by activated lymphocytes from various animal species including humans. The LT molecules within each animal species examined are heterogeneous with respect to size and charge (1). Immunological and biochemical studies in both the human, murine, and guinea pig LT systems have determined that certain but not all of the various molecular weight LT classes are an interrelated family of molecules (2, 3). Further biochemical analysis of the interrelated human LT components has revealed that one of two possible situations exists. Either the various LT forms comprise a subunit system of toxins, or the lower molecular weight (M_r) lytic components result from proteolytic cleavage of the higher M_r LT forms (4, 5). The smaller M_r forms or classes of LT, α, β, and γ (<90,000 Da) can be dissociated from the alpha heavy (α_H) (140–160,000 Da) or complex (Cx) (>200,000 Da) M_r classes of LT by changes in ionic strength or chromatography on molecular sieving columns (4, 5). The interrelated M_r LT classes appear to have different functional capacities. The low M_r forms are lytic on select target cells *in vitro*, i.e., the L-929 cell (a murine fibroblast cell line); however, the larger human and murine M_r classes are unstable but more lytically active on a wide variety of cells (6, 7). Additional findings in both the human and murine LT systems indicate that the Cx and also human α_H forms released by

[1] Supported by National Institutes of Health Grants 2 R01 AI CA 09460 and 5 T32 CA 09054.

0008-8749/83 $3.00

lectin-stimulated *immune* lymphocytes may be associated with Ig-like component(s) (6–8). These receptors are not classical immunoglobins and appear to originate from T lymphocytes.

Functional studies with human α_H (7) and Cx (6) LT forms obtained from lectin-activated *nonimmune* cells indicated that they are lytic *in vitro* for an NK-sensitive human cell line, K-562 (9, 10). Since these lymphocytes were polyclonally stimulated with lectins, the possibility exists that these forms are produced by the subpopulation of lymphocytes which mediates NK. The present studies examine the possibility that populations of human lymphocytes which have been activated *in vitro* to contain high levels of "NK-like effectors" (11, 12) produce LT forms which bind selectively to the K-562. In these studies, human lymphocytes are preactivated by coculturing human peripheral blood lymphocytes with fetal calf serum (cPBL) or mitomycin-C treated continuous lines (LCCL) for 5 days *in vitro*, and then stimulating these preactivated cells with concanavalin A (Con A). The supernatants obtained from the preactivated lymphocytes after lectin stimulation were then used to determine if cell-binding forms and cell-lytic forms are the same or if they represent discrete populations of LT molecules.

MATERIALS AND METHODS

Target cells and culture media. Target cells for standard LT assays used in these experiments were L-929 (mouse fibroblast) that were maintained as monolayer cultures in 32-oz prescription bottles in RPMI 1640 (GIBCO, Grand Island, N.Y.) supplemented with 3% heat-inactivated fetal calf serum (FCS, GIBCO), 100 U/ml penicillin, and 100 μg/ml streptomycin (RPMI-3%). Human continuous cell lines employed in these studies were K-562 (erythroid leukemia); Raji, RPMI-1788, PGLC33h, and WI-12 (B lymphoblastoid); and MOLT-4 (T-lymphoblastoid cell). Culture medium used for maintaining human continuous cell lines was RPMI 1640 supplemented with 10% FCS (RPMI-10%).

Lymphocyte cultures and supernatants. Lymphocytes were obtained from defibrinated peripheral blood by the Ficoll–Hypaque technique of Boyum (13). Interface mononuclear cells were carefully removed and washed three times with serum-free RPMI 1640 (RPMI-0%). These cells were preactivated by coculturing in either the presence of FCS (cPBL) or with mitomycin C (Sigma Chemical Co., St. Louis, Mo.)-treated continuous cell lines. The cPBL cultures were established at a density of 2×10^6 cells/ml in RPMI-10% and incubated at 37°C. The LCCL cultures were established by coculturing with mitomycin C-treated lymphoblastoid cells at a ratio of 20:1 and at a density of 2×10^6 cells/ml in RPMI-10% at 37°C. After 5–7 days, the viable lymphocytes from the cPBL and LCCL cultures were stimulated with 10–20 μg/ml Con A (Sigma) at a density of 2×10^6 cells/ml in RPMI-10% for 5, 24, or 48 hr. The supernatants were then collected by centrifugation and tested directly or stored at −20°C until use.

Lymphotoxin 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-3% containing 0.5 μg/ml mitomycin C. After a 24-hr incubation at 37°C, 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. A unit of LT activity is expressed as the

amount of LT necessary to lyse 50,000 L-cell targets. Units of LT activity in a supernatant are obtained by taking the reciprocal of the dilution of LT necessary to kill 50% of the target L cells.

^{51}Cr-Release assay. Human cell lines K-562, MOLT-4, and Raji were used as targets in these assays. All reactions were carried out in round-bottom microcytotoxicity plates (Flow Laboratories, Inglewood, Calif.) containing a total volume of 0.12 ml. Radiolabeling of target cells was accomplished by addition of 100 μl of ^{51}Na chromate, 100 μCi (305 mCi/μg, New England Nuclear Corp., Boston, Mass.), to 5×10^6 cells in 1 ml RPMI-0%. After 1–2 hr at 37°C, treated cells were washed three times by alternate sedimentation and resuspension (800*g* for 5 min) with cold RPMI-10%. Cells (10^4 in 0.02 ml) were added to microtiter wells containing 0.05 ml of sample or control media plus 0.05 ml of RPMI-10% and were incubated at 37°C for 18 hr. The release of ^{51}Cr label was measured by uptake of cell-free supernatant with Titerteks supernatant collection system (Flow Laboratories, Inglewood, Calif.) and quantitated in an automated gamma counter (Beckman Instruments, Fullerton, Calif.). The total ^{51}Cr releasable was determined by the addition of 0.1 ml of 3% (w/v) sodium dodecyl sulfate solution (Sigma), which represented 90–95% of the total counts. Spontaneous ^{51}Cr release was 1–2%/hr from the target cells.

Target cell destruction was determined using the formula

$$\% \text{ lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100.$$

The data are represented as the mean of 3–6 experimental values ± the standard deviation.

Formalin fixation of the adsorbing target cell. Cells were treated with 1 ml of 0.1% Formalin in phosphate-buffered saline (PBS), pH 7.2, at 4°C. After 30 min these cells were washed four times by alternate centrifugation and resuspension with 15-ml vol of RPMI-0% and recounted on a hemocytometer.

Adsorption assay. Adsorbing cells were washed twice in RPMI-0% and resuspended in test or control supernatants at a concentration of $25–50 \times 10^6$ cells/ml. After 20 min at 4°C, the cells were sedimented by centrifugation (800*g* for 5 min), and absorbed and unabsorbed supernatants were tested for units of cytotoxic activity on L-929 cells or on ^{51}Cr-labeled targets. The amount of LT activity in these supernatants was determined as described for the LT assay. For some samples, the percentage adsorption of LT activity was calculated using the formula

$$\% \text{ adsorption of LT activity} = \frac{(\text{U before adsorption}) - (\text{U after adsorption})}{(\text{U before adsorption})} \times 100.$$

For the ^{51}Cr-release assay, the percentage adsorption of lytic activity was calculated as

$$\% \text{ adsorption of lysis} = 1 - \frac{\%\ ^{51}\text{Cr-release for adsorbed sample}}{\%\ ^{51}\text{Cr-release for unadsorbed sample}} \times 100.$$

RESULTS

Removal of LT Activity for L-929 Cells by Incubation with K-562 Cells

Data shown in Table 1 indicate that supernatants obtained after 5 or 24 hr of lectin stimulation from cPBL or LCCL cultures contain LT activity detected on

TABLE 1

Removal of LT Activity in Supernatants from Lectin-Stimulated cPBL and LCCL by Incubation with K-562 Cells

		Time of supernatant collection[a]			
		5 hr		24 hr	
Expt.	Culture	Activity (U)[b]	Percentage removed[c]	Activity (U)	Percentage removed
1	cPBL	39	85	ND[d]	ND
2	cPBL	28	89	43	52
3	cPBL	33	54	158	52
4	cPBL	27	29	ND	ND
5	cPBL	24	50	55	80
6	cPBL	30	47	42	48
7	LCCL	31	64	ND	ND
8	LCCL	43	60	134	46
9	LCCL	75	60	ND	ND
10	LCCL	57	67	ND	ND
11	LCCL	ND	ND	69	79

[a] Supernatants were collected from preactivated lymphoid cells after 5 or 24 hr of lectin stimulation.

[b] Units of LT activity were determined on L-929 cells as described under Materials and Methods.

[c] Supernatant samples were incubated with 2.5×10^7 K-562 cells/ml for 20 min at 4°C. The cells were removed by centrifugation and the supernatant was tested for LT activity on L-929 cells. The percentage removed by the absorbing cells is calculated as described under Materials and Methods.

[d] ND, not done.

L-929 cells which is removed by incubation with K-562 cells at 4°C. While all of the data are not shown, we found adsorption with K-562 cells measured 10 or more units/ml of LT in 41 out of 54 different supernatants. The percentage LT removal in these 41 supernatants ranged from 12 to 90%. Lymphocytes obtained at various times from a single donor appear to differ in their capacity to release LT forms which are removed by K-562 cells (Table 1, Expts. 3 and 4, 5 hr; Expts. 5 and 6, 24 hr).

One sample containing 35 U (units/ml of LT activity) was divided into six aliquots and tested in parallel to examine the degree of variation in a single supernatant. The values obtained for the levels of activity removed were 16–20 U or 54 ± 5%. In addition, an aliquot of this supernatant was stored at −20°C and tested after 30 days; 14 U or 64% of the activity was removed out of 22 total units.

Analysis of the Nature of the Removal of L-929 Cell-Lytic Activity by K-562 Cells

Two types of experiments were designed to demonstrate that these molecules are binding to cells. First, we tested several aliquots of a single sample with increasing numbers of K-562 cells. The data shown in Fig. 1 indicate only a certain percentage of activity was depleted by the adsorbing cells. Also shown in Fig. 1 is the saturation curve obtained with formaldehyde-treated K-562 cells (closed circles) which was similar to that obtained with untreated cells (open circles). To examine the kinetics of binding, aliquots of a single supernatant were incubated with a constant number of K-562 cells for various intervals of time. The results shown in Fig. 2 demonstrate

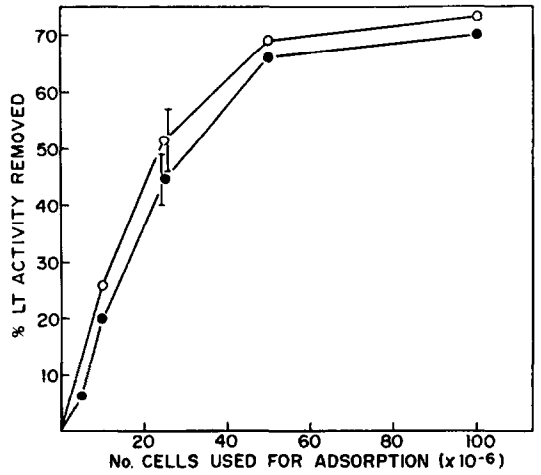

FIG. 1. Removal of LT activity from a supernatant by incubation with increasing numbers of K-562 cells. A supernatant from cPBL was collected after 5 hr of lectin stimulation. One-milliliter aliquots were incubated with various numbers of K-562 for 20 min at 4°C and then tested for LT activity on L-929 cells. The percentage removal of LT activity was calculated as described under Materials and Methods. The original sample contained 35 units of LT/ml. Open circles, untreated K-562 cells; closed circles, formaldehyde-treated K-562 cells. The error bars indicate the range of values obtained for four separate aliquots.

that removal of the majority of lytic activity occurs in less than 1 min, and the remainder of binding material is removed within 5 to 10 min.

Specificity of Binding

Supernatants from cPBL and LCCL cultures were employed to determine whether binding of LT is related to the NK sensitivity of the adsorbing cell. Several additional

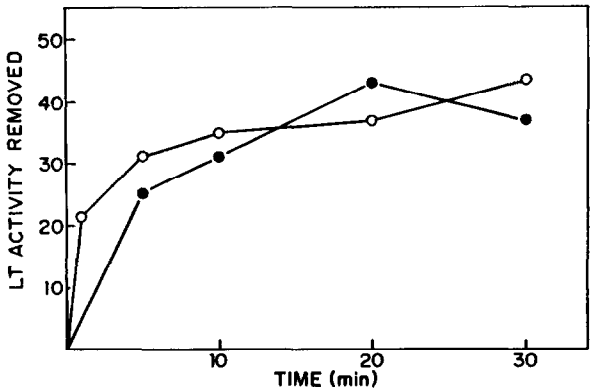

FIG. 2. Kinetics of removal of LT activity from a supernatant by incubation with K-562 cells. The vertical axis measures percentage LT activity removed. A supernatant from cPBL was collected after 5 hr of lectin stimulation. Aliquots were incubated with K-562 cells at concentrations of 25×16^6 cells/ml for various intervals of time and tested for LT activity on L-929 cells. The removal of LT units was calculated as described under Materials and Methods. The original sample contained 51 units of LT/ml. Open circles, untreated K-562 cells; closed circles, formaldehyde-treated K-562 cells.

cell lines with varying degrees of sensitivity to human NK effectors were tested for their ability to remove lytic activity from these supernatants. The results of these studies are shown in Table 2. Supernatants adsorbed with MOLT-4 (NK-sensitive) and Raji (NK-resistant) cells exhibited low levels of binding (<10 units of activity), while K-562 removed from 23 to 46 units of lytic activity. There does not appear to be a direct relationship between the levels of L-cell lytic activity bound and the NK sensitivity of these cell lines.

Lysis of Allogeneic Target Cells by Lectin-Induced Supernatants from Preactivated Human Lymphocytes

Supernatants from LCCL and cPBL were obtained after 5 hr lectin stimulation and immediately tested for their ability to induce lysis of L-929 in the standard LT assay and of MOLT-4 and K-562 cells in a ^{51}Cr-release assay (Table 3). The LT activity detected on L-929 cells in these supernatants varies from 12 to 72 units/ml. The data presented in Table 1 indicate that five out of seven supernatants from cPBL, when tested on MOLT-4 cells, result in significant levels of lysis (21–63% ^{51}Cr release) after 18 hr. While not shown, kinetic studies of cell lysis induced by these supernatants revealed that it became detectable at 6–8 hr and increased thereafter. At longer incubation times (greater than 18 hr), increased cell lysis was observed but also a high spontaneous value of ^{51}Cr release was seen. Three of the seven supernatants tested were also lytically active on K-562 target cells (11–31% ^{51}Cr release). The level of lytic activity detectable on MOLT-4 appears to correlate well with the levels of LT activity (lysis of L-929 cells) in these cPBL supernatants. Of those supernatants which contained >15 U LT activity (Expts. 1–7, Table 3), four out of five are lytic for MOLT-4 cells, and two out of five are toxic for K-562 cells. When lower levels of LT activity are obtained (Expts. 5 and 6, Table 1), MOLT-4 targets are not lysed, but in one experiment, K-562 targets were lysed (Expt. 5, Table 3).

The results of similar experiments utilizing 5-hr supernatants from lectin-stimulated LCCL cultures are shown in Table 3; four of five experiments gave significant lytic activity (20–43% ^{51}Cr release) on MOLT-4 target cells (Expts. 8, 9, 11, and 12). In contrast, only one of five supernatants tested was lytically active on K-562 targets (Expt. 9, Table 3; 31% ^{51}Cr release).

TABLE 2

Adsorption of Supernatants from cPBL and LCCL-Derived PBL with Cell Lines with Different Sensitivities to Human NK Effector Cells[a]

Expt.	Source of supernatant	Total LT (units/ml)	Percentage adsorption of each cell line		
			K-562	MOLT-4	Raji
1	cPBL	42	90	14	21
2	cPBL	47	55	23	−21
3	cPBL	72	64	12	7
4	LCCL[b]	36	64	11	3

[a] Supernatants from cPBL or LCCL-derived PBL were collected after 5 or 24 hr of lectin stimulation and tested for adsorption to various untreated cell lines as described under the LT-adsorption assay. The cell concentrations used for the adsorption studies were 50×10^6 cells/ml of supernatant.

[b] The stimulating cell line used in the LCCL was WI-L2.

TABLE 3

Capacity of Supernatants from Lectin-Stimulated cPBL and LCCL to Cause Lysis of Various Target Cell Lines[a]

Expt.[b]	Cell source of supernatant	LT activity L cells	Percentage ^{51}Cr release	
			MOLT-4	K-562
1	cPBL	72	56 ± 4	11 ± 3
2	cPBL	47	63 ± 7	28 ± 4
3	cPBL	42	21 ± 8	3 ± 2
4	cPBL	37	22 ± 6	1 ± 4
5	cPBL	15	9 ± 7	31 ± 8
6	cPBL	14	3 ± 2	3 ± 4
7	cPBL	ND[c]	41 ± 6	1 ± 4
8	LCCL	42	28 ± 6	4 ± 4
9	LCCL	15	43 ± 14	31 ± 3
10	LCCL	12	3 ± 4	6 ± 4
11	LCCL	ND	30 ± 10	2 ± 1
12	LCCL	ND	20 ± 8	0 ± 8

[a] The cPBL and LCCL were cultured in RPMI-10% for 5–7 days and then stimulated with Con A for 5 hr as described under Materials and Methods. Supernatants were collected and tested for (1) LT activity detected on L-929 cells and (2) lysis of ^{51}Cr-labeled MOLT-4 and K-562 target cells in 18 hr.

[b] Different supernatants were tested in each experiment. Nine different blood donors were used; two donors were used multiple times, one donor was used for Expts. 3, 6, and 9; and the other donors were used for Expts. 2 and 8.

[c] ND, not done.

Stability of Supernatant Lytic Activity for MOLT-4 Target Cells to Storage

Several supernatants were tested on MOLT-4 target cells immediately after production, stored at −20°C, and retested for lytic activity after various time intervals (Table 4). These studies utilized 5-hr supernatants from both cPBL- and LCCL-treated cells. Six of seven supernatants were active on MOLT-4 cells by Day 1; only three

TABLE 4

Stability of Supernatant Lytic Activity for MOLT-4 Target Cells to Storage at −20°C[a]

Sample	Source of 5-hr supernatant	Length of storage (days)	Percentage ^{51}Cr release from MOLT-4 targets	
			Before storage	After storage
1	cPBL	2	13 ± 2	11 ± 9
2	cPBL	3	26 ± 6	48 ± 8
3	LCCL	4	20 ± 8	8 ± 1
4	cPBL	4	7 ± 9	14 ± 4
5	cPBL	6	41 ± 6	1 ± 2
6	LCCL	7	30 ± 10	−1 ± 1
7	cPBL	7	21 ± 8	3 ± 1

[a] Supernatants were generated from cPBL or LCCL-derived PBL (RPMI 1788 stimulator cells) by stimulating with Con A for 5 hr. These were tested for lysis of ^{51}Cr-labeled MOLT-4 target cells in an 18-hr assay. Separate aliquots were stored at −20°C and tested after various intervals.

had detectable levels of lytic activity after storage for 2–7 days. The supernatant with the highest level of lytic activity after storage (sample 2, Table 4) was not lytically active on MOLT-4 cells when tested on Day 10. However, additional experiments revealed that freeze–thawing of fresh supernatants does not affect lytic activity.

The Relationship between Cell-Lytic Forms for MOLT-4, K-562 Cells, and K-562 Binding LT-Lytic Forms for L-929 Cells

These supernatants contained LT-lytic forms for L cells which bind to K-562 cells. Experiments were designed to examine the relationship between these binding K-562 forms and lytic forms for MOLT-4 and K-562 cells. Aliquots of each culture supernatant were tested for levels of LT activity and K-562-binding forms on L-929 targets. The results shown in Table 5 demonstrate that all five supernatants tested had lytic activity for MOLT-4 and L-929 cells, and only two of five supernatants contained significant levels of lytic activity for the K-562 cells (Expts. 1 and 2). Yet supernatants 3 and 4 possessed good LT activity and K-562-binding forms, with no apparent lytic effect on K-562 targets. The same supernatants were also tested for lytic activity on MOLT-4 and K-562 cells. This observation indicates that LT forms detected on K-562 and L-929 cells may not necessarily be the same materials.

Adsorption of Supernatants with K-562, MOLT-4, and Raji Cell Lines Removes Lytic Activity for MOLT-4 and K-562 Cells

Supernatants from 5-hr Con A-stimulated cPBL and LCCL cultures were adsorbed with MOLT-4, K-562, or Raji and then tested for lytic activity on MOLT-4 cells in an 18-hr ^{51}Cr-release assay. The levels of lysis of MOLT-4 cells by the adsorbed aliquots of each sample were compared to the level of lysis by the unadsorbed sample. The results of these adsorption studies are shown in Table 6. Four of the six supernatants tested had lytic forms which were equally removed by adsorption with any

TABLE 5

The Relationship of Supernatant Lytic Activity for MOLT-4 and K-562 Target Cells to the Presence of LT Forms That Bind to K-562 Cells[a]

Expt.	Cell source of supernatant	Percentage ^{51}Cr release		LT activity (U)	LT activity bound to K-562 cells[b]
		MOLT-4 cells	K-562 cells		
1	cPBL	63 ± 7	28 ± 4	47	+(55)[c]
2	cPBL	56 ± 4	11 ± 3	72	+(64)
3	LCCL[d]	27 ± 6	4 ± 4	42	+(40)
4	cPBL	21 ± 8	3 ± 2	42	+(90)
5	cPBL	22 ± 6	1 ± 4	37	—

[a] Supernatants were generated from cPBL or LCCL-derived PBL by stimulation with Con A for 5 hr. Each supernatant was tested for (1) lysis of ^{51}Cr-labeled MOLT-4 target cells in 18 hr (2) lysis of ^{51}Cr-labeled K-562 target cells in 18 hr, and (3) LT activity against L-929 cells. In addition, separate aliquots were adsorbed on K-562 cells at 50×10^6 cells/ml of supernatant, and then tested on L-929 cells for LT activity. Cells used for adsorption in Expts. 3 and 4 were Formalin-treated.

[b] +, ≥10 U bound; −, <10 U bound.

[c] Percentage adsorption of LT activity in parentheses.

[d] RPMI 1788 stimulator cells.

TABLE 6

The Effect of Adsorption of Supernatants with MOLT-4, K-562, and Raji Cell Lines on Lytic Activity for MOLT-4 Target Cells[a]

Sample	Cell source of supernatant	LT units	Percentage ^{51}Cr release from MOLT-4 target cells			
			Unadsorbed sample	Adsorbed to MOLT-4	Adsorbed to K-562	Adsorbed to Raji
1	cPBL	47	63 ± 7	35 ± 5 (45)[b]	23 ± 3 (61)	29 ± 5 (54)
2	cPBL	72	56 ± 4	36 ± 4 (36)	32 ± 9 (43)	29 ± 1 (48)
3	LCCL	36	43 ± 14	−2 ± 4 (104)	4 ± 1 (91)	2 ± 6 (95)
4	LCCL	42	27 ± 6	33 ± 5	21 ± 5	23 ± 4
5	cPBL	ND[c]	22 ± 6	34 ± 3	25 ± 8	33 ± 10
6	cPBL	ND	14 ± 4	4 ± 1 (71)	−4 ± 4 (107)	3 ± 2 (79)

[a] Supernatants were generated from cPBL or LCCL-derived PBL (RPMI 1788 stimulator cells) as described under Materials and Methods. Aliquots of each supernatant were adsorbed to three different cell lines at 50 × 10^6 cells/ml of supernatant. These adsorbed aliquots and the untreated supernatants were then tested for lytic activity on ^{51}Cr-labeled MOLT-4 target cells. Cells used for adsorption of samples 3, 5, and 6 were Formalin-treated.

[b] Percentage adsorption of lytic activity in parentheses.

[c] ND, not done.

of the above cell lines (Expts. 1–3 and 6). Each cell line removed an equivalent level of lytic activity for MOLT-4 cells with each sample tested.

Supernatants containing lytic activity for K-562 (Expts. 1 and 2; Table 7) were adsorbed on MOLT-4, K-562, and Raji cell lines and then tested for lytic activity on K-562 cells in an 18-hr ^{51}Cr-release assay. The results of these experiments are shown in Table 7. The adsorbing cell lines, MOLT-4, K-562, and Raji, all removed lytic forms for the K-562 cells. The percentage of activity removed was approximately the same within each sample.

DISCUSSION

The present studies reveal that human LT activity can be adsorbed from supernatants by incubation with native or Formalin-treated target cells at 4°C. The LT-

TABLE 7

The Effect of Adsorption of Supernatants with MOLT-4, K-562, and Raji Cell Lines on Lytic Activity for K-562 Target Cells[a]

Sample	Percentage ^{51}Cr release from target cells			
	Unadsorbed sample	Adsorbed to MOLT-4	Adsorbed to K-562	Adsorbed to Raji
1	28 ± 4	2 ± 2 (93)[b]	0 ± 4 (100)	2 ± 7 (93)
2	21 ± 3	1 ± 2 (91)	4 ± 2 (81)	2 ± 3 (89)

[a] Supernatants were generated from cPBL and adsorbed to various cell lines as described for Table 6. The adsorbed aliquots and untreated supernatants were then tested for lytic activity on ^{51}Cr-labeled K-562 target cells. Cells used for adsorption were untreated.

[b] Percentage adsorption of lytic activity in parentheses.

containing supernatants were generated from human PBL which were preactivated by culture in medium containing FCS or with mitomycin C-treated allogeneic lymphoblastoid cells. Lymphoid cells preactivated in this fashion have been shown to gain increased NK-like cell-lytic ability and also to be capable of rapid LT release from presynthesized intracellular pools (15). The percentages of LT activity removed from supernatants by adsorption with K-562 cells are compared for 41 supernatants; they ranged from 12 to 90%. Several factors may be responsible for the range in the levels of K-562-binding forms present in different supernatants: (a) different cell donors, (b) variations of various effector cells within the donor lymphocyte population, (c) changes in the sensitivity of the assay system, and (d) binding forms may be degraded into nonbinding components, which are still lytically active on L-929 cells. However, the major proportion of this variation does not appear to be due to experimental manipulation, because the range of values obtained for aliquots of a single sample assayed in the same day in parallel is very small. The LT activity detected on L-929 cells which is removed by K-562 cells is stable in storage at −20°C and can still be detected after 30 days.

Lymphotoxins in these supernatants are binding to absorbing cells and not being degraded or internalized. Several types of studies were performed to eliminate these possibilities. First, removal of lytic activity by adsorbtion with the K-562 cells occurs at 4°C, a temperature which is inhibitory for most enzymes and cellular processes. Second, when aliquots of a supernatant are incubated with different numbers of K-562 cells, the amount of LT adsorbed from a supernatant reaches a plateau, and this plateau is below 100% removal. This observation indicates that only a certain discrete proportion of the LT forms can bind to the K-562 cell and therefore does not reflect nonspecific enzymatic degradation or internalization. Third, Formalin-treated K-562 cells bind equivalent amounts of LT as untreated or native K-562 cells. The removal process, therefore, is independent of enzymes and cellular events which are inactivated by formaldehyde. Finally, binding occurs very rapidly, i.e., within minutes. Collectively, these data support the concept that LT activity is binding to a determinant(s) which is(are) present on the surface of the K-562 cell.

Supernatants containing LT forms detected on L-929 cells which bind to K-562 cells were tested for binding to several additional cell lines. These studies revealed that when the levels of binding are compared to the levels bound to the K-562, the percentages of activity bound to the other cell lines are much lower and do not correlate to their sensitivity as targets for NK–effector cells. Thus, the K-562 is unique in that it binds the largest proportion of L-cell-lytic LT activity. However, this does not appear to be the case for the NK-sensitive cell line MOLT-4, a human T-cell line, that was found to be much less efficient than the K-562 at removing lytic activity.

Supernatants from cPBL and LCCL cultures obtained by secondary stimulation with a lectin for 5 hr were tested for cell-lytic activity using three different target cell lines, L-929, MOLT-4, and K-562, and a 16-hr ^{51}Cr-release assay. Of the two NK-sensitive cell lines, K-562 and MOLT-4, MOLT-4 cells are more sensitive to lysis induced by these supernatants than are the K-562 cells. These results are similar to those reported by Wright and Bonavida (16), in which MOLT-4 cells were found to be more sensitive to lysis *in vitro* than K-562 cells to soluble mediators produced by freshly isolated human PBL stimulated by a lectin-coated L-929 cell monolayer. We found lysis of MOLT-4 target cells by supernatants from cPBL is protracted. No lysis can be detected at 5 hr of incubation; however, significant levels are detectable after

12 hr, and levels of cytotoxicity climb higher after 18 hr. The degree of lysis may actually continue to increase after this time interval, but later time points were not examined because of the high levels of spontaneous ^{51}Cr release from the target cells after 18 hr. An examination of the stability of supernatant lytic activity from MOLT-4 and K-562 cells revealed that, for most supernatants, these lytic materials are not stable to storage at −20°C for more than a few weeks. However, L-929 cell-lytic activity in these same supernatants can be recovered after storage under the same conditions for very long periods.

Experiments were designed to determine if the L-929 cell-lytic forms which bind to K-562 cells are related to the lytic activity detectable on ^{51}Cr-labeled MOLT-4 or K-562 target cells. The results of these studies indicate that there is no correlation between the presence in a supernatant of MOLT-4 or K-562 cell-lytic LT activity and the presence of K-562-binding forms of LT detected on L-929 cells. These supernatants clearly contain multiple LT forms with different cell-binding and cell-lytic capacity. Clearly, K-562 and, to a lesser extent, MOLT-4 express receptors for a certain population of forms which lyse the L-929 cell. In contrast, the Raji does not express any receptor(s) for L-929 cell-lytic forms. However, MOLT-4, K-562, and Raji all possess receptors for LT forms which have cell-lytic activity in descending order on the same cells. These studies support the concept that LT forms are heterogeneous, both in their binding and in cell-lytic capacity. It should be noted that a low level of L-929 and MOLT-4 cell-lytic activity did not bind to any target.

The cPBL- and the LCCL-prestimulated lymphocytes have been shown by *in vitro* techniques to contain several types of nonspecific, "NK-like," and anomalous killer effector cells (11, 12, 17); the LCCL also generates T lymphocytes which are immune to the allogeneic stimulator cells (18). The present studies employ a short secondary polyclonal lectin stimulus which induces different populations of the preactivated lymphocytes to rapidly release LT from preformed pools into the supernatant (15). The presence of LT forms with different cell-binding and lytic activity in these supernatants raises the possibility they may be of polyclonal origin, and studies with defined lines of different types of effector cell are underway in attempts to answer this interesting question. These experiments reveal that supernatant-induced lysis of MOLT-4 cells, as shown from lysis of L-929 cells, can now also be divided into at least two steps: (a) a cell-binding phase and (b) the actual process of cell lysis. Moreover, these studies also indicate that a cell may be resistant to lysis, but still express receptors for these cell toxins. It is tempting to speculate that the reason why some supernatants have high cell-lytic activity and others low activity, is that binding forms may be more lytically active than nonbinding forms. It is important to note here, as mentioned previously, that no single target cell, for example, the L-929 cell, is uniformly sensitive to all LT forms, and different targets are essential to detect different forms of LT activity (19).

REFERENCES

1. Ross, M. W., Tiangco, G. J., Horn, P., Hiserodt, J. C., and Granger, G. A., *J. Immunol.* **123,** 325, 1979.
2. Granger, G. A., Yamamoto, R. S., Fair, D. S., and Hiserodt, J. C., *Cell. Immuno.* **38,** 388, 1978.
3. Hiserodt, J. C., Tiangco, G. J., and Granger, G. A., *J. Immunol.* **123,** 317, 1979.
4. Hiserodt, J. C., Yamamoto, R. S., and Granger, G. A., *Cell. Immunol.* **41,** 380, 1978.
5. Harris, P. C., Yamamoto, R. S., Crane, J., and Granger, G. A., *J. Immunol.* **126,** 2165, 1981.

6. Yamamoto, R. S., Hiserodt, J. C., and Granger, G. A., *Cell. Immunol.* **45,** 261, 1979.
7. Harris, P. C., Yamamoto, R. S., Christensen, C., and Granger, G. A., submitted for publication.
8. Hiserodt, J. C., Tiangco, G. J., and Granger, G. A., *J. Immunol.* **123,** 332, 1979.
9. Bonnard, G. D., and West, W. H., *in* "Immunodiagnosis of Cancer" (R. B. Herberman and K. R. McIntire, Eds.), p. 1032. Dekker, New York, 1978.
10. Hansson, M., Bakacs, K. K. T., Kiessling, R., and Klein, G., *J. Immunol.* **121,** 6, 1978.
11. Jondal, M., and Targan, S., *J. Exp. Med.* **147,** 1621, 1978.
12. Zielske, J. V., and Golub, S. H., *Cancer Res.* **36,** 3842, 1976.
13. Boyum, A., *Scand. J. Immunol.* **5,** 9, 1976.
14. Spofford, B., Daynes, R. A., and Granger, G. A., *J. Immunol.* **112,** 2111, 1974.
15. Ware, C. F., Harris, P. C., and Granger, G. A., *J. Immunol.* **126,** 1427, 1981.
16. Wright, S. C., and Bonavida, B., *J. Immunol.* **126,** 1516, 1981.
17. Tomonari, K., *J. Immunol.* **124,** 1111, 1980.
18. Sharma, B. S., and Terasaki, P. I., *Cancer Res.* **34,** 115, 1974.
19. Devlin, J. J. Yamamoto, R. S., and Granger, G. A., *Cell. Immunol.* **61,** 22, 1981.