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THE LT SYSTEM IN EXPERIMENTAL ANIMALS

I. Rapid Release of High Levels of Lymphotoxin (LT) Activity from Murine Lymphocytes during the Interaction with Lectin-Treated Allogeneic or Xenogeneic Target Cells *in Vitro*[1]

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High levels of cytotoxic activity (lymphotoxin-like (LT)) detectable on L-929 cells was obtained in serum-free culture supernatants when non-adherent murine splenic lymphocytes or nylon wool-enriched T cells were cultured on monolayers of mitogen- (PHA) coated allogeneic or xenogeneic stimulator cells *in vitro*. Levels of lytic activity were lower in supernatants obtained from splenocyte cultures containing glass-adherent cell populations. Although release of lytic activity was very rapid, reaching maximal levels by 6 to 10 hr, this activity was very unstable. The levels of toxic activity in 8-hr supernatants were 20 to 50 times the levels obtained when lymphocytes were cultured with various dosages of mitogen (PHA-P, Con A) alone, even after 5 days of incubation. This phenomenon was not unique to murine lymphoid cells, for similarly high levels of LT activity were found in supernatants from lymphoid cells obtained from several animal species activated in a similar fashion. These results indicate that lymphoid cells from several animal species are capable of rapidly releasing high levels of cell-lytic activity *in vitro* not previously noted, and provide a means for obtaining highly active supernatants for biochemical studies. Furthermore, the data suggest that rapid release of LT may depend upon the nature of the cellular activating stimulus involving interaction of the lymphocyte with both lectin and target cell surface.

Several laboratories have reported on the biochemical characteristics of lymphotoxins (LT)[2] released *in vitro* by activated lymphoid cells from experimental animals and man (1–7). However, these studies have been difficult because of technical problems in obtaining sufficient quantities of highly active supernatants, for these molecules are generally present in supernatants in very low levels (4, 8) and certain forms are highly unstable (2, 9). Most studies, to date, have focused on human LT molecules, because culture techniques have been devised that yield relatively high levels of these materials in a stable or semi-stable form (9, 10), and they are released by selected continuous human lymphoid cell lines (11, 12).

The routine method employed by most investigators for obtaining LT activity from activated lymphoid cells *in vitro* is to incubate the cells with one of a variety of mitogens or antigens for 1 to 5 days and then collect the culture supernatants. In comparative studies, we have found that mitogen-activated human lymphocytes release up to 100 times more LT than murine lymphoid cells stimulated under similar conditions (13). This manuscript reveals a new method for inducing the *rapid* release of *high* levels of cell-lytic (LT-like) activity from activated lymphoid cells from mice and several other animal species. Supernatants from experimental animals can be collected in 8 to 10 hr, and can be produced in protein-free media, such that biochemical studies can be more easily approached. A major problem we encountered is that the activity in murine supernatants is quite unstable, thus, supernatants must be handled rapidly.

MATERIALS AND METHODS

1. Culture media and cell lines. Culture media employed in these studies consisted of RPMI 1640 supplemented with 3% heat inactivated (56°C, 60 min) fetal calf serum (FCS) (GIBCO, Grand Island, New York) 20 μg/ml streptomycin and 100 units/ml penicillin (1640-S). An LT-sensitive substrain of L-929 cells developed and maintained in our laboratory (14) were obtained from stock monolayer cultures and grown in 32-oz prescription bottles at 37°C, in a 95% air, 5% CO_2 atmosphere, and passed biweekly. Murine 3T3 and human HeLa cells were a generous gift of Dr. Dennis Cunningham, Department of Microbiology, University of California, Irvine, and were grown similar to L-929 cells, except media were supplemented with 10% FCS.

2. Preparation of murine spleen cells. Spleens from C57BL/6, DBA/2, or C3H/DiSn 8 to 10-week-old mice (Jackson Labs, Bar Harbor, Maine), or spleens from various other animal species, were aseptically obtained, and a single cell suspension prepared in RPMI 1640-10% FCS, as described previously (15). Cell viability was determined by exclusion of 0.1% Eosin Y and was routinely 90 to 100%. The lymphoid cell suspensions were

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[1] This research was supported by Grant 1882 from the Rheumatic Diseases Research Foundation; Grant IM-32 from the American Cancer Society; and Grant AI-09460 from the Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland.
[2] Abbreviations used in this paper: BS, boiled serum, a heat stable serum fraction; Cx, complex, a high m.w. LT form; DEAE, diethylaminoethyl cellulose-11; HS, high salt; LS, low salt; LT, lymphotoxin; NA lymphocytes, nonglass-adherent splenic lymphocytes; PAGE, polyacrylamide gel electrophoresis; PHA/L cells, phytohemagglutinin-P coated L-929 fibroblasts; PHA-P, phytohemagglutinin-P; PS, physiologic salt; PWM, pokeweed mitogen.

routinely placed in 250-ml plastic T flasks (Falcon, Oxnard, Calif.), or 16-oz glass prescription bottles at a density of approximately 5×10^6 cells/ml, for 1 to 2 hr at 37°C to remove adherent cells. The nonadherent cells were then collected, washed, and resuspended to a final density of 2.5×10^6 cells/ml for use in these studies. Differential and viable cell counts revealed these cells were 96 to 98% lymphocytes and 90 to 100% viable.

3. Separation of splenic T lymphocytes. Enriched T lymphocyte populations were obtained from nylon wool columns as described by Julius (16). Briefly, 10^8 nonadherent splenic lymphocytes in 2 ml 1640-5% FCS were slowly eluted into a 12-ml plastic syringe containing 1 g of acid-washed nylon wool (leukopak) packed to the 9-ml mark. These cells were allowed to incubate on the column for 1 to 2 hr at 37°C. The column was then washed with 50 ml 1640-5% FCS, and the eluting cells (T cells) were collected. Cell recovery after this procedure was generally 20 to 25% of the input cells, and cell viability was routinely 90%. The purity of these T lymphocytes was determined by fluorescence staining by employing a commercially available FITC conjugated polyspecific rabbit anti-mouse Ig sera (Cappel, Dowington, Pa.). Cells staining positive with this sera in four separate experiments were 41 to 48% in the unseparated spleen and 3 to 6% in the T cell-enriched population.

4. Production of LT-containing culture supernatants. 5×10^5 L-929, BALB 3T3, or human HeLa cells were established in 90-ml plastic T flasks in RPMI-S 10 to 20 hr before use. After this time period, the media were poured off and these monolayers were treated with phytohemagglutinin-P (PHA-P, Difco, Detroit, Mich.) at 100 μg/ml/10^5 cells in 1640-S or control 1640-S for 1 hr at 37°C. In all experiments, PHA-P was freshly prepared before use. The medium was again poured off, the monolayers were washed twice with PBS, and 10 ml of a cell suspension containing 25×10^6 lymphoid cells in 1640-S were added. After various times at 37°C, the supernatants were collected, cleared of cells by centrifugation (500 × G, 10 min) and immediately tested for LT activity or frozen at −70°C.

5. Lymphotoxin assay. The amount of LT in a given supernatant was determined as previously described (17). Briefly, various amounts (0.05 to 0.20 ml) of each supernatant were placed in duplicate 1-ml tube cultures of L-929 cells containing 100,000 mitomycin-C treated L cells per tube. Sixteen to 24 hr later the medium was discarded, and the remaining viable adherent L cells were enumerated in a Coulter Counter. The highest dilution that killed 50% of the cells was then determined. One unit of LT activity is defined as that amount of LT that will destroy 50% of the cells (50,000) in these tubes. The reciprocal of the dilution giving 50% destruction provided the number of units present per milliliter in the original undiluted supernatant.

RESULTS

1. Capacity of C57BL/6 murine spleen cells to release LT-like activity when placed on lectin-coated allogeneic cells in vitro. In previous studies from this laboratory, it was usual to incubate murine lymphoid cells from normal or immune animals with activating lectins or antigens, or with lectin-coated target cell monolayers for periods of 2 to 5 days *in vitro*, then to collect the LT-containing medium (13). Activity in these supernatants was usually low and averaged from 1 to 10 units/ml. However, during experiments studying target cell lysis in which lymphocytes were allowed to interact with lectin-coated target L-929 cells *in vitro*, we noticed that shortly after lymphocyte-target L cell interaction, high levels of supernatant LT-like activity was detectable. Although the present manuscript does not show physical proof that the cell lytic activity in these early supernatants is due to lymphotoxin(s), data to prove this is the case will be presented in the second and third papers of this series. To investigate further this phenomenon, L-929 cell cultures of C3H origin were established in T flasks and coated with PHA-P or left untreated, as described in *Methods*. They were then incubated with nonadherent spleen cells from normal C57BL/6 mice at a 50 to 1 lymphocyte to stimulator cell ratio (final spleen cell density was 2.5×10^6 cells/ml). In addition, spleen cells from these mice were cultured at various cell densities in the presence of various amounts of PHA-P or Con A, but in the absence of stimulator cells. The culture supernatants were collected at different time intervals, cleared of cells by centrifugation, frozen at −70°C, and all tested for LT activity at the end of each experiment. A representative result of five similar experiments is shown in Figure 1. It is evident that there is a very rapid release of toxic activity into the supernatant, which was detectable within 4 to 6 hr after interaction of lymphocytes with monolayers of PHA-P coated L cells *in vitro*. Although the levels of LT activity present in these supernatants reached a peak in 8 to 10 hr, they rapidly declined over the next 10 hr to a constant low level. In contrast, supernatants obtained from splenic lymphocytes cultured with PHA-P or Con A alone and tested over this same time interval (0 to 24 hr) never contained more than 5 to 10 units of LT activity/ml. In fact, stimulation of murine lymphocytes with various concentrations of PHA-P over a full 5 days never resulted in the release of more than 5 to 10 units of activity/ml (data not shown). It is also clear from the data shown in Figure 1 that levels of cell lytic activity reached a peak as stimulator L-929 cell destruction began, and declined rapidly as lysis progressed.

2. Capacity of nonadherent (NA) spleen cells or enriched T

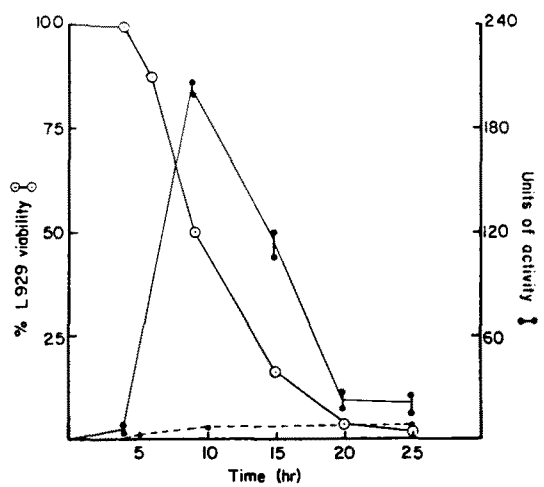

Figure 1. Nonadherent C57BL/6 murine spleen cells were incubated at a 50:1 ratio with PHA-coated L-929 cells, noncoated L-929 cells, or with 20 μg/ml PHA-P alone. After various intervals, the supernatants were harvested, cleared of cells by centrifugation, and frozen at −70°C. After all supernatants had been collected, they were simultaneously tested for LT activity, as described in the text. The number of units of LT activity/milliliter in each sample is depicted on the ordinate, and the time the sample was collected on the abscissa. The number of remaining viable L-929 target cells in each Petri dish was also determined by washing the monolayers three times with PBS (this removed 98% of the attached lymphocytes), trypsinizing the L-929 cells, and counting in a Coulter Counter. ⊙——⊙, % PHA-treated target L-929 cell viability; ●——●, units of supernatant LT activity/ml; ○——○, units of LT activity/ml detected in cultures activated with PHA-P (20 μg/ml) and no targets.

cell populations to release LT activity in vitro. It has previously been reported that cells adherent to glass or plastic within a lymphoid cell population can rapidly and spontaneously release in culture nonspecific cell toxin(s) (18–20). To investigate the possible role of these cells and their products in this phenomenon, we removed them by absorption, as described in *Methods.* The results of four separate experiments are shown in Table I. These data reveal that murine spleen cell populations, when separated from glass-adherent cells (even after two sequential absorptions on glass), released significantly higher levels of cell-lytic activity into the supernatant when incubated with lectin-coated stimulator L-929 cells than when unseparated. Thus, we routinely removed the glass adherent cells in order to optimize maximal toxin(s) release. Furthermore, the data shown in Table I indicates that isolated nylon wool enriched C57BL/6 or DBA/2 T cells (which were 4% Ig-positive lymphocytes) could release high levels of LT activity in this system.

3. Effects of lymphocyte density or the presence of serum in culture on the release of cell-lytic activity by C57BL/6 lymphoid cells in vitro. Normal C57BL/6 nonadherent splenic lymphoid cells were placed on 5×10^5 PHA-coated or noncoated L-929 stimulator cells at ratios varying from 2:1 to 200:1 (final lymphocyte densities of 2×10^5/ml to 2×10^7/ml) for 8 hr at 37°C. The supernatants were then collected and immediately tested for cell-lytic activity. Shown in Figure 2 are the representative results of two identical experiments. Clearly, maximal release of lytic activity occurs at lymphocyte:stimulator cell ratios between 25:1 and 50:1, or final lymphocyte densities of 1.25×10^6/ml and 2.5×10^6/ml, respectively. In addition, the data show that lymphocytes placed on non-PHA coated L-929 cells also released cell-lytic activity, but the levels were lower than the amount detected when spleen cells are placed on PHA coated L-929 cells. In other experiments cultures were established to determine the effect of serum on the release of high levels of cell lytic activity. In no case did the presence or absence of serum (up to 10% FCS) affect the final levels of LT activity detectable in these supernatants (data not shown).

4. Effect of various lymphocyte-target cell strain combina-

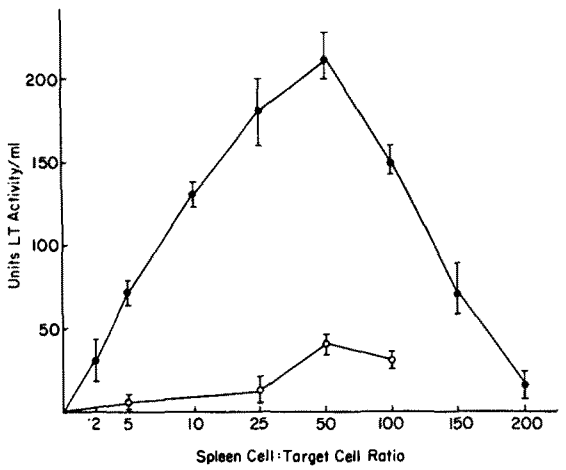

Figure 2. Nonadherent C57BL/6 murine spleen cells were incubated at various lymphocyte to target cell ratios with L-929 target cells coated with PHA-P or left uncoated. After 8 hr at 37°C, the supernatants were collected and immediately tested for toxic activity as described in *Materials and Methods.* ●—●, PHA-coated L-929 cells; ○—○, uncoated L-929 cells.

TABLE I

Rapid release of LT activity (units/ml) from C57BL/6 or DBA/2 NA lymphocytes or purified T cells on lectin-coated allogeneic L-929 monolayers in vitro[a]

Expt. No.	Lymphoid Cells	Stimulation	LT Activity			
			4 hr	8 hr	15 hr	20 hr
			units/ml			
1	C57BL/6 spleen cells	None	4	8	3	3
	Unseparated	L-929	4	28	14	5
		PHA-coated L-929	11	62	26	10
1	C57BL/6 NA spleen cells	L-929	4	69	22	6
	Absorbed 2 ×	PHA-coated L-929	19	138	59	20
	Unseparated	PHA-coated L-929	4	85	50	15
2	DBA/2 NA spleen cells	L-929	5	56	20	2
	Absorbed 1 ×	PHA-coated L-929	22	119	47	15
	Unseparated	PHA-coated L-929	3	65	28	7
3	C57BL/6 T lymphocytes	PHA-P (20 μg/ml)	4	6	8	
		L-929	2	22	9	2
		PHA-coated L-929	8	54	33	10
	Unseparated	PHA-coated L-929	3	36	15	7
4	DBA/2 T lymphocytes	L-929	6	29	8	
		PHA-coated L-929	18	109	50	
	Unseparated	PHA-coated L-929	4	65	30	

[a] Normal C57BL/6 or DBA/2 splenic lymphoid cells were obtained, and the plastic adherent cells were removed after one absorption (1 ×) or after two sequential absorptions (2 ×), as described in *Materials and Methods.* The resulting NA lymphocytes or nylon wool purified T lymphocytes (96%—Expt. 3; 97%—Expt. 4) were then placed on monolayers of L-929 fibroblasts that were coated with PHA-P or left untreated, as described in *Materials and Methods.* Other cultures received PHA-P alone at 20 μg/ml or were left untreated. After various periods of time, the cellfree supernatants were collected and immediately tested for toxic activity on mitomycin C-treated L-929 cells. Standard deviation for each data point shown is consistently less than 10%.

tions on the release of cell-lytic activity in vitro. Experiments were also conducted to determine if this observation was a general phenomenon and would occur with aggressor lymphoid cells obtained from different inbred mouse strains and various types of stimulator cells. Thus, spleen cells from C57BL/6, DBA/2, or C3H/DiSn mice were allowed to interact with lectin and nonlectin-coated L-929 (H-2^k) 3T3 (H-2^d), or HeLa (human) cells, as described in *Methods.* The supernatants were collected at 8 and 24 hr and assayed for LT activity. The representative results of four separate sets of experiments in this series are also shown in Table II. As can be seen, high levels of supernatant lytic activity were detected when nonglass-adherent spleen cells of various mouse strains were cultured with various types of PHA-P coated stimulator cells *in vitro.* In contrast, supernatants obtained from nonadherent donor lymphocytes, mixed with nonlectin treated stimulator L cells, had lower levels of toxic activity, however, this activity was higher than that detectable in supernatants of lymphoid cells co-cultured with lectin alone (Fig. 1). Additional experiments shown in Table III revealed that nonadherent spleen cells obtained from murine, rat, guinea pig, rabbit, or hamster could also be induced to release high levels of lytic activity by 8 hr after interaction with PHA-coated L-929 monolayers *in vitro.*

5. Evidence that toxic activity in these cultures is lymphocyte derived. To eliminate the possibility that toxic activity in these cultures was due to agents such as hydrolytic enzymes released by dead or dying target cells, or nonspecific intracellular enzymes released by polymorphonuclear cells (PMN), monocytes, or lymphocytes, two separate types of experiments were performed: 1) to assess whether this activity was derived from dying target cells, various numbers of murine L-929, 3T3, or human HeLa cells were suspended in 2 ml of RPMI 1640 and completely lysed by sonication. After the cell debris was removed by centrifugation at 12,000 × G, for 30 min, the supernatants were collected and assayed for toxic activity on L-929 cells. As can be seen in Table IV, essentially no detectable lytic activity is obtained from homogenates of these cells, even at a concentration of 50×10^6 cells/ml (100 times the number used in these experiments); 2) to assess whether this toxic activity was derived from nonspecific intracellular enzymes present in PMN cells, monocytes, or lymphocytes, various numbers of unseparated normal C57BL/6 spleen cells were suspended in 2 ml of RPMI 1640, sonicated (>99% lysis), the cell debris was removed by centrifugation as above, and the supernatant was tested for toxic activity. Also shown in Table IV are the results of this experiment. Clearly, intracellular lytic activity is *very* low (<2 units/ml) when 500×10^6 spleen cells (>100 times the number used/ml in these experiments) are lysed in a volume of 2 ml.

TABLE II

Release of high levels of LT activity by NA C57BL/6, DBA/2, or C3H/DiSn murine spleen cells activated on lectin-coated allogenic or xenogeneic target cells in vitro[a]

Source of Effector Cells	Target Cells	Lectin Coated	Units LT Activity/ml	
			8 hr	24 hr
C57BL/6 (H-2^b)	L-929 (H-2^k)	+	*120 ± 20*	24 ± 5
C57BL/6 (H-2^b)	L-929 (H-2^k)	−	30 ± 8	15 ± 2
C57BL/6 (H-2^b)	3T3 (H-2^d)	+	*95 ± 12*	10 ± 6
C57BL/6 (H-2^b)	3T3 (H-2^d)	−	27 ± 4	9 ± 3
C57BL/6 (H-2^b)	HeLa (human)	+	*114 ± 12*	20 ± 5
C57BL/6 (H-2^b)	HeLa (human)	−	30 ± 3	10 ± 1
C3H/DiSn (H-2^k)	L-929 (H-2^k)	+	*75 ± 9*	28 ± 1
C3H/DiSn (H-2^k)	L-929 (H-2^k)	−	29 ± 3	10 ± 2
C3H/DiSn (H-2^k)	3T3 (H-2^d)	+	*80 ± 2*	30 ± 4
C3H/DiSn (H-2^k)	3T3 (H-2^d)	−	18 ± 2	N.T.[b]

[a] NA cells from normal C57BL/6, DBA/2, or C3H/DiSn mice were obtained and placed on various target cell monolayers, which were coated with PHA-P or left untreated (Ly to target ratio = 50:1). After incubation for 8 or 24 hr at 37°C, the supernatants were collected and tested for LT activity, as described in *Materials and Methods.* Error values represent standard deviation for triplicate determinations of a single point.

[b] N.T., not tested.

TABLE III

Detection of high levels of LT activity in supernatants of lymphoid cells from various animal species cultured on PHA-coated L-929 monolayers in vitro[a]

Animal Species	LT Activity		
	4 hr	8 hr	24 hr
		units/ml	
Mouse (C57BL/6)	21 ± 2	108 ± 6	15 ± 1
Guinea pig	45 ± 2	320 ± 15	300 ± 21
Rat		350 ± 10	
Hamster	70 ± 5	265 ± 18	
Rabbit (New Zealand White)	46 ± 5	190 ± 12	

[a] NA lymphoid cells obtained from various animal species were incubated on monolayers of PHA-P coated L-929 cells in RPMI-S, as described in *Materials and Methods.* After various periods of time at 37°C, the supernatants were collected and tested for toxic LT activity on mitomycin C-treated L-929 cells as described in *Materials and Methods.*

TABLE IV

Toxic activity in homogenates of L-929 cells, HeLa cells, or C57BL/6 murine spleen cells on L-929 cells in vitro[a]

Cell Type	No. of Cells Employed	Toxic Activity
		units/ml
L-929	10^7	0
L-929	5×10^7	<1
HeLa	5×10^7	<1
Unseparated C57BL/6 spleen cells	5×10^7	<1
Unseparated C57BL/6 spleen cells	5×10^8	2

[a] Various numbers of L-929 or HeLa cells were obtained from stock monolayer cultures, washed, suspended in 2 ml of RPMI-1640, and lysed by sonication. Homogenates of unseparated C57BL/6 spleen cells were prepared in an identical manner. Microscopic observations revealed in all cases virtually 100% cell lysis had occurred. The homogenates were then centrifuged 12,000 × G, 30 min, and the resulting supernatants were tested for toxic activity on L-929 cells as described in *Materials and Methods.*

DISCUSSION

These data reveal that murine lymphoid cells are capable of rapidly releasing high levels of cytotoxic activity detectable on L-929 cells *in vitro* when activated on lectin-coated cell monolayers. Although LT activity could be detected in these cultures within hours, the levels reached a maximum of about 200 units/ml in 8 to 10 hr, then declined to low levels, about 10 to 20 units/ml by 24 hr. Such rapid release and high levels of LT activity detectable in supernatants from murine lymphoid cells has not been previously observed. However, this was a general phenomenon, because rapid appearance of high levels of LT activity was also observed when lymphoid cells from several other animal species were activated on lectin-coated xenogeneic L-929 cells *in vitro.*

The advantages of the present system over previously employed culture systems for obtaining highly active supernatants are obvious: First, collection of supernatants within a few hours after lymphocyte activation minimizes contamination of these supernatants with intracellular components from dead or dying lymphoid cells. Second, cultures can be established in serum-free media, such that biochemical studies can be more easily approached. Third, collection of early supernatants minimizes loss of unstable molecules that may decay or be degraded during several days in culture. Due to previously noted low levels of LT activity in 3- to 5-day cultures of lectin-activated murine lymphoid cells, it was originally thought that these cells were not capable of releasing substantial amounts of soluble phase LT (13). This clearly does not appear to be the case. In fact, these cells *can* release high levels of LT activity, the magnitude and speed of which appears to be controlled by the nature of the "triggering" events occurring at the lymphocyte cell surface.

Extensive studies revealed that the lytic activity detected in these cultures was not due to toxic material(s) released by glass-adherent cells, PMN cells, or dead L cells, but is derived from lymphoid cells. This was established by direct testing of homogenates of C57BL/6 murine spleen cells, L-929 cells, 3T3 cells, or HeLa cells, which would contain nonspecific intracellular "toxic materials" and could be mistaken as LT. These homogenates were virtually nontoxic to L-929 cells even at levels that were up to 100 times the numbers of cells employed in these studies. These findings corroborate similar studies in the human and mouse in which little or no cytolytic activity detectable on L-929 cells was present in homogenates of unactivated lymphoid cells (1, 21). Furthermore, significantly higher levels of LT were measured when glass-adherent cells were removed from these preparations, an observation that may suggest glass-adherent cells have some type of regulatory role. Although nylon wool column-enriched murine T lymphocytes were capable of rapid LT release in this system, other lymphoid cell types also appear to be able to release in these cultures. The data are not shown, however, we have found that spleen cells from T-deficient nude mice and spleen cells from normal mice treated with anti-θ serum and complement also release toxic activity in this system. Thus, it appears this is a situation where different lymphoid cell types are stimulated to release these molecules. It is interesting to note, however, that although this system works well with spleen cells from various animal species, it is not effective when human peripheral blood lymphoid cells are employed.

There may be several possible reasons for the rapid decline in LT activity in these supernatants: First, as target cells undergo lysis, release of cellular material(s) (membrane proteins, proteases, etc.) may inactivate the LT activity. In this regard, Lies (22) has reported that membrane products isolated from murine L-929 cells can dramatically effect soluble human LT activity *in vitro*. Furthermore, Kobayashi *et al.* (23) have isolated a membrane glycoprotein(s) from L-929 cells that appears to inhibit guinea pig LT activity detectable on L-929 cells *in vitro*. Second, soluble phase LT molecules may be highly unstable and spontaneously lose their biologic activity. This is a serious problem, for supernatants must be handled very rapidly in order to achieve biochemical fractionation. Furthermore, extensive biochemical studies on molecules with LT activity present in these supernatants reveal these molecules are larger m.w. forms, not previously noted (6, 13). Thus, it is conceivable that enhanced lytic activity observed in these early supernatants is *not* due to more LT molecules, but more highly active larger m.w. forms that rapidly decay or dissociate into smaller m.w. and less lytically active molecules. Third, the lymphocytes themselves release component(s) that inactivate these materials. In fact, there is precedent for this possibility, for we have previously reported that human lymphocytes release a product(s) that can inactivate β class human LT (9). These may be important mechanism(s) for degrading soluble phase LT molecules. Finally, the presence of glass-adherent cells clearly influences the levels of LT activity detected. The mechanism of how this occurs is at present not clear.

These studies emphasize the fact that the type of activating event occurring at the lymphocyte membrane surface is directly related to the amount and speed of release of LT into the supernatant. In previous and present studies, treatment of murine lymphocytes with lectin alone induced release of only very low levels of LT activity *in vitro*. However, lectin bound on stimulator cell surfaces induced rapid, high level LT release when exposed to lymphocytes. Although the highest levels of LT release were consistently seen in cultures of nonglass-adherent lymphocytes cultured with lectin-coated stimulator cells *in vitro*, lymphocytes cultured with stimulator cells alone also released LT activity. It appears that the nature of the stimulating agent(s) on the surface of the target cell, when presented to a lymphocyte, may regulate the amount of LT release. This finding also appears to be a general one, for lymphoid cells obtained from many different animal species can release high levels of LT activity when stimulated in this manner. Furthermore, there is a requirement for cell:cell interaction and multiple signals to induce rapid LT release, for only very low levels were detected when lymphocytes were "activated" with PHA coupled to Sepharose beads, and syngeneic lymphocyte stimulator cell combinations even in the presence of lectin do not induce LT release (unpublished results). However, an alternative explanation for these results is that lectin-coated stimulator cell monolayers enhance LT production by providing a nonspecific "feeder layer".

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