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Stabilization and Functional Studies of High-Molecular-Weight Murine Lymphotoxins

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High levels of lymphotoxin-like activity (LT) were found in supernatants from secondarily stimulated immune mouse splenocytes activated with concanavalin A (Con A) *in vitro*. Splenocytes obtained from C57Bl/6 mice immune to the P815 mastocytoma were restimulated *in vitro* with mitomycin C-treated P815 cells, and then stimulated with Con A. High levels of unstable LT activity are rapidly (2-4 hr) released by these lectin-stimulated splenocytes. The introduction of a crosslinking agent, glutaraldehyde, was found to stabilize this LT activity and allowed us to perform more defined biochemical studies and to examine the functional activities of the LT classes. The lytic activity in these supernatants resided in the high-molecular-weight classes, termed Complex (Cx > 200,000 daltons) and alpha-heavy (α_H , 130,000-160,000 daltons). It was found that the Cx and α_H LT classes from the secondarily stimulated immune splenocytes cause lysis of allogeneic target cells, P815 and EL-4, in a 16-hr ^{75}Se -methionine release assay, and in some cases, this lysis was specific for the sensitizing target cell.

INTRODUCTION

Lymphokines are a family of biologically active molecules released by immune and nonimmune lymphocytes, when they are stimulated *in vitro* by antigens or lectins. One lymphokine, lymphotoxin (LT), has been shown to inhibit cell growth and cause target cell lysis *in vitro* (1, 2). Murine lymphocytes release various molecular weight classes of LT that can be resolved by molecular sieving, termed Complex (Cx, >200,000 daltons), alpha-heavy (α_H 130,000-160,000 daltons), alpha (α , 70,000-90,000 daltons), beta (β , 35,000-50,000 daltons), and gamma (γ , 15,000-20,000 daltons), (3, 4). These same molecular weight classes have been observed in supernatants from activated lymphocytes from many animal species that have been studied (5).

Physical-chemical and immunological studies have demonstrated that the Cx and α_H classes are composed of the LT forms (α , β , and γ), indicating that these classes are interrelated. Under conditions of high-ionic-strength buffers, Cx and α_H from several animal species dissociate into toxins similar to the lower-molecular-weight forms (3, 5, 6). In the case of human, murine, and guinea pig LT, antisera have been raised against a smaller-molecular-weight LT class which also neutralizes the larger-molecular-weight forms (3, 5, 7). It has been proposed that the small LT forms may be either subunits or proteolytic fragments of the larger molecules.

Recent evidence indicates Cx and α_H LT molecules, but not the smaller forms, may be associated with nonclassical antigen-binding receptors. Human Cx and α_H classes are neutralized by antisera to the Fab region of human IgG molecules, while antisera to the constant region of IgG or IgM heavy chains did not cause significant inhibition (6, 8, 9). In addition, lytic activity in supernatants from both human and murine alloimmune lymphocytes can be specifically adsorbed by the allogeneic cell line used for stimulation (10, 11). Also, it has recently been reported that LT released by lymphocytes from alloimmunized mice causes the specific lysis *in vitro* of the tumor cell line used for immunization (11). The report of antigen-specific cell-lytic forms of murine LT potentially originating from cytotoxic T lymphocytes was interesting; however, lysis of the tumor cells by the LT-containing supernatants was low, rarely exceeding 20%, even after being concentrated 5 to 20 times. In addition, these forms were difficult to study because of their extreme instability.

We have developed a new *in vitro* method to produce highly toxic LT forms from alloimmune lymphoid cells. We are able to stabilize the lytic activity with the crosslinking agent glutaraldehyde. These advances have allowed preliminary studies of both the biochemical and functional activities of individual molecular weight classes of murine LT.

MATERIALS AND METHODS

Culture medium and cell lines. Culture medium employed in these studies consisted of RPMI 1640 supplemented to either 3% heat-inactivated fetal calf serum (FCS) (RPMI-3%) or 10% FCS (RPMI-10%) (Gibco, Grand Island, N.Y.), 100 $\mu\text{g}/\text{ml}$ streptomycin, and 100 units/ml penicillin. An LT-sensitive substrain of L-929 cells (L cells) developed and maintained in our laboratory, was grown in 32-oz prescription bottles at 37°C, in 95% air, 5% CO₂ atmosphere, and passed bi-weekly in RPMI-3% (12). Murine suspension cell lines P815 (H-2^d) and EL-4 (H-2^b) were grown in RPMI-10% under the same conditions as the L cells.

Immunization protocol and harvesting murine spleen cells. The mice used were 8- to 12-week old C57Bl/6 (H-2^b) (ARS, Madison, Wisc.). Animals were immunized by intraperitoneal injection of 10⁷ P815 mastocytoma. After 21 days, the spleens were aseptically removed, and a single-cell suspension was prepared. Spleen cells from nonimmune animals were routinely placed in 32-oz bottles at a density of 5 × 10⁶ cells/ml for 1 to 2 hr at 37°C in medium containing 10% FCS, to remove glass-adherent cells.

In vitro restimulation of immune splenocytes. Spleen cells from immune animals were restimulated *in vitro* by P815 cells which had been treated with mitomycin C (Sigma Chemical Co. St. Louis, Mo) (50 $\mu\text{g}/10^7$ cells) for 1 hr at 37°C, and washed three times with RPMI. These cells were cultured at 5 × 10⁶ cells/ml with a ratio of 25:1 lymphocyte to tumor cells for 4 days (13). At the end of the 4 days, the cells were harvested by centrifugation at 300g for 5 min and employed in these studies.

Production of cell-lytic culture supernatants. Production of nonimmune LT-containing supernatants has been described previously (14). Briefly, 10⁸ nonadherent normal murine splenic lymphoid cells were placed on phytohemagglutinin P (PHA-P, Difco, Detroit, Mich.)-coated L-929 cells (4 × 10⁶) in RPMI-10% at

a density of 2.5×10^6 cells/ml. These cultures were then incubated 6–8 hr at 37°C at which time the culture supernatants were collected.

To generate immune LT, splenic lymphoid cells from a 4-day culture were re-suspended at a density of 2×10^6 viable cells/ml and stimulated with PHA-P or concanavalin A (Con A, Sigma), $20 \mu\text{g/ml}$ in RPMI 1640 supplemented to 1% FCS for 2–24 hr at 37°C . At the end of each of the incubation periods, the supernatant was collected and cells were removed by centrifugation at $1000g$ for 10 min.

Treatment of LT-containing supernatant with glutaraldehyde. LT containing supernatants were treated with 25% glutaraldehyde (Sigma) so that the final concentration of glutaraldehyde was 0.025% at 25°C for 1 hr.

Lymphotoxin assay. Two types of assay employing the L cell were used. Supernatants were either tested on L cells to determine the number of units per milliliter of LT activity, or a fixed amount from each column fraction was assayed on L cells, and the number of viable cells remaining after 24 hr was used to determine where toxicity was eluting from molecular sieving columns. These techniques are described in detail elsewhere (6, 15).

Physical-chemical separation of LT. The methods employed for fractionation of LT activity in supernatants from activated murine lymphocytes were identical to those employed for separated human LT molecules, all of which have been previously published (3, 5). All procedures were performed at 4°C . Briefly, 2-ml samples of $25\times$ concentrated murine LT supernatant containing from 500 to 5000 units of LT activity were chromatographed on molecular sieving columns containing Ultrogel AcA 44 (LKB, Bromma, Sweden). One of the columns used was 90×2.4 cm, from which we collected 6.5-ml fractions; the other column was 60×2.4 cm and 4.7 ml fractions were collected. Aliquots of the eluted fractions of between 0.05 and 0.2 ml were assayed on L-929 cells for LT activity. The molecular weight markers are described in Fig. 1.

$^{75}\text{Se-Met}$ release assay. P815 and EL-4 cell lines used as targets in these studies were radiolabeled using a modification of the procedure of Brooks *et al.* (16). Approximately 4×10^6 cells were incubated at 37°C overnight in 10 ml of RPMI-10% containing $20 \mu\text{Ci}$ of ^{75}Se -methionine ($^{75}\text{Se-Met}$) (Amersham, Arlington Heights, Ill.). Cells were then washed four times ($800g$ for 6 min) with RPMI-3%. Cells were resuspended in RPMI-10% and 10^4 cells in 0.02 ml added to each well of a round-bottom microcytotoxicity plate (Flow Laboratories, Inglewood, Calif.). All wells contained a total volume of 0.22 ml, consisting of either 3% sodium dodecyl sulfate to determine total releasable counts, RPMI-1%, to determine spontaneous release, LT containing media, or effector cells from *in vitro* cultures. A 4-hr assay was used to test cell-mediated lysis, and a 16-hr assay was used for LT-mediated lysis. Target cell destruction was determined with the following formula:

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

RESULTS

Stabilization of Murine LT by Glutaraldehyde

The instability of murine LT led to a series of attempts to stabilize the lytic activity. Numerous methods were tried, but we were only successful with the cross-

linking reagent glutaraldehyde. The level of glutaraldehyde used in this work was determined from a dose curve of the effect of various concentrations of glutaraldehyde on LT activity. It was found that 0.025% glutaraldehyde resulted in retention of lytic activity for at least three weeks at 4°C. To remove the toxicity due to glutaraldehyde, the treated supernatants were either dialyzed or chromatographed over an Aca 44 molecular sieving column.

Supernatants containing nonimmune murine LT in RPMI-3% were split into two samples. Glutaraldehyde was added to one sample, and the other was left untreated. Both samples were then concentrated and chromatographed over an Aca 44 molecular sieving column, and assayed for toxicity on L cells (Fig. 1A). The treated sample shows more toxicity in the column profile. This is because the toxicity in the untreated sample has been decaying in the time between supernatant collection and assay of the column fraction. If a sample of supernatant is assayed immediately, it is just as toxic as a glutaraldehyde-treated sample. The major peak of toxicity in both samples eluted in the 130,000- to 160,000 molecular-weight range, which is characteristic of α_H . The fractions were stored at 4°C for 2 weeks and reassayed (Fig. 1B). All lytic activity in the untreated sample was lost during storage, while the treated supernatant retained toxicity. Control medium containing

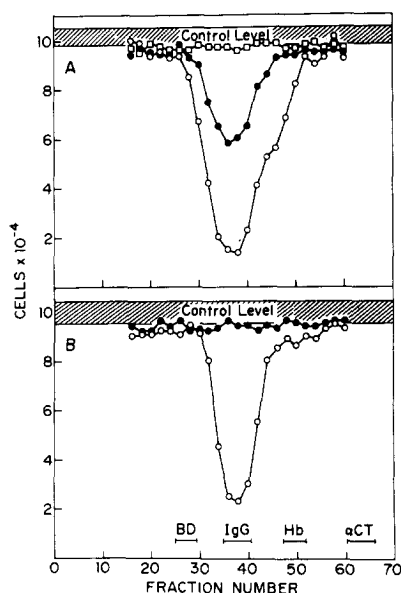


FIG. 1. Molecular sieving chromatography of murine supernatant on Aca 44. The number of viable L cells remaining after the fractions were assayed is given on the ordinate. The elution profiles of various molecular weight markers are indicated by horizontal bars. They are: blue dextran (BD) $>2 \times 10^6$ MW; human IgG, 150,000 MW; Hemoglobin (Hb), 63,000 MW; and α -chymotrypsin (α -Ct), 23,000 MW. The 90×2.4 -cm columns were equilibrated and eluted in 10 mM phosphate buffer, containing 10^{-4} M EDTA. (A) Murine LT-containing supernatants or RPMI-3% were either treated with glutaraldehyde at a final concentration of 0.025%, or left untreated, concentrated 17 \times , and chromatographed over an Aca 44 column, and then assayed in duplicate on L-929 cells as described under Materials and Methods. The duplicates varied no more than 6% (\square) Glutaraldehyde-treated RPMI-3%; (\circ) glutaraldehyde-treated murine LT; (\bullet) nonglutaraldehyde-treated murine LT. (B) These same murine LT-containing supernatants as in A were stored at 4°C for 3 weeks and reassayed on L-929 cells for cytotoxic activity. (\circ) Glutaraldehyde-treated murine LT; (\bullet) nonglutaraldehyde-treated murine LT.

3% FCS and glutaraldehyde, when treated in an identical manner, was not toxic (Fig. 1A).

Treatment with glutaraldehyde did not detectably alter the molecular weight of these supernatant LT forms. Glutaraldehyde is known to form covalent crosslinks between lysine residues in proteins (17). However, even in the presence of 3% FCS, intermolecular crosslinking did not seem to occur, as the peak of toxicity eluted in the same fraction in both the treated and nontreated sample (Fig. 1A).

Glutaraldehyde treatment did not make RPMI-3% toxic or detectably change the molecular weight of the toxic molecules in these supernatants. This indicates that preexisting LT is being stabilized rather than glutaraldehyde forming new toxins. Additionally, glutaraldehyde does not increase the toxicity of supernatants (data not shown).

Glutaraldehyde Reacts Directly with LT Molecules

Glutaraldehyde stabilization of cell-lytic activity appears to be a direct action on the LT molecules, rather than the inactivation of a separate degradative enzyme. To demonstrate this, we glutaraldehyde-treated a supernatant containing 100 units/ml of nonimmune LT activity. FCS was added to a final concentration of 10%, and the sample was dialyzed overnight to remove remaining reactive glutaraldehyde. A sample of this treated supernatant was then mixed with an equal volume of fresh untreated supernatant (which also had an LT activity of 100 units/ml). After 48 hr, the treated supernatant retained its original activity, while the activity in the untreated supernatant had fallen to <10 units/ml. The mixture of treated and fresh untreated LT had slightly more than half the original activity after 48 hr. Thus it seems that the activity due to the untreated LT in the mixture was lost, but the activity from the treated LT remained. So it would appear that the untreated supernatant did not contain a degradative enzyme responsible for loss of activity. This experiment was repeated three times with similar results.

Kinetics of Immune LT Production

The production system employed in this study generates LT in half the time required by procedures previously used in this laboratory (14). Figure 2 shows LT activity in the supernatant reaching a maximum in 8–10 hr, when alloimmune splenocytes are stimulated on a PHA-coated monolayer of L cells, as was done previously. However, we found that immune lymphocytes, restimulated *in vitro*, released peak levels of LT in just 3–4 hr, when placed on a lectin-coated monolayer of L cells. In an effort to avoid contamination of supernatants with material from lysed L cells, studies were performed to examine the action of lectin alone. We found Con A stimulation led to the release of maximal levels of LT within 3–4 hr in the absence of L-929 cells (Fig. 2). PHA alone would also stimulate rapid release, but not as reproducibly as Con A. In agreement with previous work (14), Con A would not stimulate rapid release of LT from alloimmune splenocytes which had not been restimulated *in vitro* by the immunizing cell line.

Molecular weight classes of immune LT. A 60 × 2.4-cm column was used for molecular weight characterization of immune LT. This column allowed us to work with these supernatants quickly, yet still gave adequate separation of the molecular weight markers. The lytic activity from these supernatants chromatographed as a

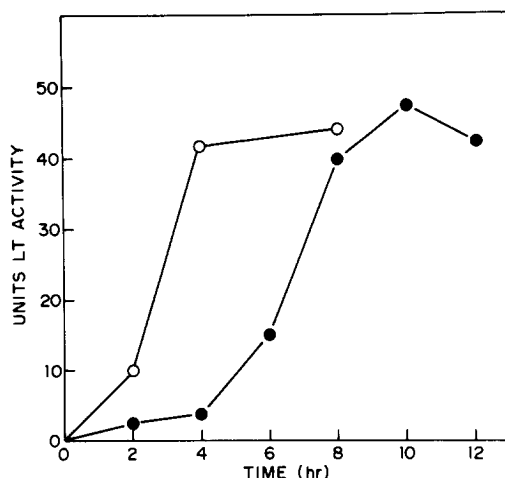


FIG. 2. Immune lymphocytes were cultured with P815 cells *in vitro* and stimulated with Con A, 10 $\mu\text{g}/\text{ml}$, at a density of $2.5 \times 10^6/\text{ml}$, or nonadherent nonimmune C57Bl/6 murine spleen cells were incubated at a 25:1 ratio with PHA-coated L-929 cells. After various time intervals, the supernatants were harvested, cleared of cells by centrifugation, and frozen at -70°C . After all the supernatants were collected, they were simultaneously tested for LT activity on L-929 cells for LT activity. Triplicate values varied by no more than 5%. The number of units of LT activity per milliliter in each sample is depicted on the ordinate, and the time the sample was collected on the abscissa. (○) Restimulated lymphocytes; (●) nonimmune C57Bl/6 murine spleen cells.

broad peak with approximately 70% of the lytic activity, as determined on L cells, present in the α_{H} class. A shoulder of high-molecular-weight toxicity eluted in the void volume of the column. This was in contrast to nonimmune LT, where we rarely find Cx forms. Fractions from the void volume of the column were pooled and termed Cx. Fractions eluting with the molecular weight marker IgG (150,000 daltons) were pooled and termed α_{H} .

Glutaraldehyde-Treated Cx and α_{H} from Alloimmune C57Bl/6 Lymphocytes Lyse both P815 and EL-4 Targets

Alloimmune lymphocytes restimulated with P815 cells for 4 days *in vitro* were assayed on ^{75}Se -Met labeled P815 and EL-4 target cells. Lysis of EL-4 cells never exceeded 5%, and 45–60% lysis of the P815 targets was observed (Table 1). The resensitized lymphocytes were then stimulated for 3 hr with Con A. These supernatants were immediately assayed on L-929 cells, and the number of units per milliliter of LT activity was determined (Table 1). The supernatants were then glutaraldehyde-treated and chromatographed, and the Cx and α_{H} fractions pooled, concentrated, and dialyzed against medium. At this time, 200 μl of these fractions was assayed on 20 μl of ^{75}Se -Met-labeled P815 or EL-4 targets for a 16-hr assay.

Table 1 presents the results of seven separate experiments. These experiments, using column fractions, demonstrated that both Cx and α_{H} LT classes are able to mediate the lysis of P815 and EL-4 targets. It is clear that lymphocyte supernatants from different cultures can contain both highly toxic Cx and α_{H} forms, while in other instances, only one MW class would lyse the ^{75}Se -Met-labeled targets.

In general, the more units of LT used in the assay, the higher the level of lysis

TABLE I

Capacity of Immune C57Bl/6 Anti-P815 Lymphocytes and Various MW LT Classes Obtained from these Lymphocytes to Induce Lysis of Allogeneic Target Cells *in Vitro*

Expt No.	Cell mediated lysis ^a		Fraction lysis ^b				LT activity ^c		
	P815	EL-4	of P815		of EL-4		Supernatant	Cx	α_H
			Cx	α_H	Cx	α_H			
I	45	2	30	19	40	19	100	41	98
II	ND ^d	ND	6	7	11	0	10	4	9
III	ND	ND	45	35	22	53	>100	>45	>108
IV	50	3	32	0	0	0	23	9	22
V	54	2	0	8	0	14	ND	ND	ND
VI	ND	ND	95	60	0	0	95	42	100
VII	60	5	0	0	0	0	91	32	76

^a Percentage lysis of P815 and EL-4 radiolabeled target cells by *in vitro* restimulated C57Bl/6 anti-P815 effector cells as described under Materials and Methods. Percentage release are expressed as the mean of triplicate values, and the standard deviations are within 5% of the mean. A 25:1 effector to target ratio was used in all cases.

^b The Cx and α_H MW LT classes were obtained from fractioned glutaraldehyde-treated supernatants derived from lectin-stimulated *in vitro*-restimulated murine lymphocytes as described under Materials and Methods. 200 μ l of either Cx or α_H was incubated with the radiolabeled allogeneic target cells for 16 hr, percentage release calculated by the formula given under Materials and Methods, and the triplicate values exhibited <10% variation.

^c Supernatants were obtained from lectin-stimulated MLC murine lymphocytes and immediately assayed on L-929 cells to determine units of LT activity/ml. The LT units/200 μ l of the Cx and α_H fractions were calculated using the concentration factors, the dilution by the column, and the distribution of toxicity in the column profile.

^d ND, not done.

of P815 and EL-4 targets. In addition, the number of units/200 μ l did not correlate with the specificity of the LT. However, it is possible that L cells are not equally sensitive to both Cx and α_H LT molecules (see Discussion). This caveat precludes the use of LT units, as measured on L cells, as a basis for a definitive quantitative comparison of the amount of lysis Cx and α_H LT cause on P815 and EL4 targets.

The column fractions from the different cultures also varied in target specificities. These data do not unequivocally support either antigen-specific lysis or nonspecific lysis; indications of both are seen. Further work will determine if the source of this variability is the lymphocyte populations or whether Cx and α_H can exist in both antigen-specific and nonspecific stages.

DISCUSSION

Stabilization of these high-molecular-weight murine LT forms with glutaraldehyde is a technical breakthrough which will permit biochemical studies of these materials. The lytic activity of these untreated forms has a 24-hr half-life, and the half-life of antigen-specific toxicity for allogeneic tumors is less than 1 hr. Thus, without stabilization, the lytic activity due to these forms is lost, leaving only the stable lower-molecular-weight forms normally found when longer production sys-

tems are used (4, 18). We have tried numerous techniques to stabilize murine LT lytic activity, e.g., storage at different temperatures and pH, the addition of polycations, protein carriers, glycerol, and protease inhibitors. None of these techniques were successful. Previous biochemical studies of murine LT forms suggested some of the instability of these molecules may be due to dissociation of Cx and α_H forms into the weakly active smaller forms (3, 5, 11). With this possibility in mind, we attempted crosslinking fresh supernatants with glutaraldehyde. The present results demonstrated that we are able to maintain the high levels of LT lytic activity as assayed on L cells for several weeks at 4°C. In further biochemical studies of these materials, the use of reversible crosslinkers should be very useful.

The stabilizing effect of glutaraldehyde is apparently due to its direct action on the LT molecule(s). Inactivation of an external factor(s) by glutaraldehyde, e.g., a degradative enzyme, appears to be unlikely, because the addition of fresh untreated supernatant did not inactivate the glutaraldehyde-treated LT. Some of the remaining possible mechanisms of stabilization of the LT molecules by glutaraldehyde are: (i) intramolecular crosslinking, preventing dissociation of subunits or denaturation; (ii) inactivation of a portion of the LT molecule which functions as an autolytic enzyme; or (iii) masking site(s) on the LT molecule recognized and clipped by a degradative enzyme. It seems that intermolecular crosslinking of LT to other components in these supernatants did not occur, for had other materials been crosslinked to the LT, the increased molecular weight would be expected to alter the elution profile of the treated LT.

Studies by Hiserodt *et al.* suggested that the forms in these supernatants, which lysed cell lines other than the L cell, were the high-molecular-weight classes (11). However, because of the unstable nature of this lymphokine(s), only minimal testing of the different molecular weight classes of LT on allogeneic target cells, other than the L cell, was performed. Stabilization of murine LT has allowed us to further study the functional activity of molecular weight column fractions. It is now obvious that both Cx and α_H are able to cause high levels of lysis when assayed on both P815 and EL-4 targets. When the fractions of the molecular sieving columns are assayed, the majority of the lytic activity, as determined by L cells, resides in the α_H pool. When assayed on P815 or EL-4 cells, however, the Cx pool contained as much if not more toxicity than the α_H pool. This could be due to the P815 and EL-4 cells being more sensitive to Cx than α_H . Alternatively, L cells may be less sensitive to Cx than α_H . This cannot be resolved in the absence of method to quantitate LT on a molar basis. Thus, the various molecular weight classes of LT may differ in their ability to lyse any particular cell line.

There is evidence to suggest that column fractions are more lytically active than unfractionated supernatants, for when supernatants were used at the same concentration as the α_H or Cx fractions, they rarely caused greater than 10% lysis on the P815 or EL-4 targets (data not shown). We obtained these results regardless of whether we used the present production method, including glutaraldehyde stabilization, or the method previously used in our laboratory to produce LT from alloimmune murine lymphocytes (14). This finding suggests that an inhibitor may be present in the whole supernatant which is removed by the molecular sieving columns. Alternatively, these supernatants may be activated by passage over the column. This observation may explain past difficulties in demonstrating lysis of various cell lines with unfractionated supernatants and suggests a mechanism for

limiting the activity of these forms to the microenvironment between effector and target cell(s).

The high levels of lysis caused by Cx and α_H LT led us to compare the lytic capacities of these column fractions with that of intact lymphocytes. For example, in one experiment the number of lymphocytes used to generate the LT for each well of microtiter plate was equivalent to a 375:1 effector to target cell ratio. In an 8-hr assay, the α_H fraction from these lymphocytes caused 46% lysis of the P815 targets, so approximately 10-fold more lymphocytes and an assay twice as long were required for α_H to cause the same level of lysis as that found in intact lymphocytes. While LT-mediated lysis is obviously less effective than the intact lymphocyte, it cannot be excluded as a possible mediator of lysis, for a variety of reasons. For example, degradation of LT before glutaraldehyde treatment, adsorption to surfaces during concentration and chromatography, and splitting the LT into separately assayed Cx and α_H pools could all reduce the total LT activity recovered from the stimulated lymphocytes. Additionally, it is known that LT is expressed on activated lymphocyte membranes (19), and that cell contact is required for cell-mediated lysis. Thus, LT may only be delivered in a highly active form during cell contact, when a high local concentration on the membrane of the effector could be delivered to a target, or LT may require a lipid environment for full activity.

The LT-mediated lysis of the target cells did not always display the pattern of specificity observed when intact lymphocytes were assayed on the target cells. Only in Experiments IV and VI was there a marked preferential lysis of the P815 targets by the fractionated LT. In the other four positive experiments, two yielded strong lysis and two gave weak lysis of both targets. It is possible that low levels of activity and instability kept this nonspecific LT below the threshold of detection in previous work, which only reported specific lysis (14). Alternatively in the present study, glutaraldehyde may have stabilized some of the LT in a nonspecific form, which was not detected previously.

The stabilization of murine LT reported in this study has removed one of the most inconvenient facets of this experimental system. Already stabilization has allowed us to demonstrate that the larger-molecular-weight forms are capable of both selective and nonselective lysis.

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REFERENCES

1. Ruddle, N. H., and Waksman, B. H., *Science* **157**, 1060, 1968.
2. Granger, G. A., and Kolb, W. P., *J. Immunol.* **101**, 111, 1968.
3. Hiserodt, J. C., Tiangco, G. J., and Granger, G. A., *J. Immunol.* **123**, 317, 1979.
4. Trivers, G., Baumgart, D., and Leonard, E. J., *J. Immunol.* **117**, 130, 1976.
5. Ross, M. W., Tiangco, G. J., Horn, P., Hiserodt, J. C., and Granger, G. A., *J. Immunol.* **123**, 325, 1979.
6. Hiserodt, J. C., Yamamoto, R. S., and Granger, G. A., *Cell. Immunol.* **38**, 417, 1978.

7. Yamamoto, R. S., Hiserodt, J. C., Lewis, J. E., Carmack, C. E., and Granger, G. A., *Cell. Immunol.* **38**, 403, 1978.
8. Harris, P., Yamamoto, R. S., Christensen, C., and Granger, G. A., *J. Immunol.*, submitted for publication.
9. Ware, C. F., Klostergaard, J., Toth, K. M., and Granger, G. A., *Cell. Immunol.*, submitted for publication.
10. Hiserodt, J. C., Yamamoto, R. S., and Granger, G. A., *Cell. Immunol.* **41**, 380, 1979.
11. Hiserodt, J. C., Tiangco, G. J., and Granger, G. A., *J. Immunol.* **123**, 332, 1979.
12. Kramer, S. J., and Granger, G. A., *Cell. Immunol.* **15**, 57, 1975.
13. Brunner, K. T., and Cerottini, J.-C., In "Progress in Immunology" (B. Amos, Ed.), p. 385. Academic Press, New York, 1971.
14. Hiserodt, J. C., Tiangco, G. J., and Granger, G. A., *J. Immunol.* **123**, 311, 1979.
15. Spofford, B., Daynes, R. A., and Granger, G. A., *J. Immunol.* **112**, 2111, 1974.
16. Brooks, C. G., Rees, R. C., and Robins, R. A., *J. Immunol. Methods* **21**, 111, 1978.
17. Peters, K., and Richards, F. M., *Annu. Rev. Biochem.* **46**, 523, 1977.
18. Kolb, W. P., and Granger, G. A., *Cell. Immunol.* **1**, 122, 1970.
19. Hiserodt, J. C., Ware, C. F., Harris, P. C., and Granger, G. A. *Cell. Immunol.* **34**, 326, 1977.