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Lymphocyte *in Vitro* Cytotoxicity: Characterization of Mouse Lymphotoxin

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This communication describes some of the physical and chemical characteristics of mouse lymphotoxin (MLT), a cytotoxic factor released by mouse lymphocytes *in vitro* after stimulation with phytohemagglutinin. Mouse LT activity was found to be heat and pH stable exhibiting properties characteristic of a protein having a molecular weight of approximately 90–150,000. The differences and similarities between mouse and human lymphotoxins are also discussed.

INTRODUCTION

Studies concerning the mechanism of lymphocyte-induced tissue destruction have been facilitated by the development of *in vitro* techniques initiated by Govearts (1) and by Rosenau and Moon (2, 3). Three basic systems in which lymphocytes have been shown to induce cellular destruction have emerged from these studies: (1) Lymphocytes obtained from animals immunized with normal or neoplastic cells cause specific destruction of donor monolayer cells (4–6); (2) nonimmune lymphocytes cause destruction of target cells in the presence of various mitogens which are able to induce lymphocyte transformation, e.g., phytohemagglutinin (PHA) (7–9); (3) lymphoid cells demonstrating delayed-type hypersensitivity cause the nonspecific destruction of target cells when incubated with the sensitizing antigen (10). All of these reactions appear to be initiated by the interaction of antigen or mitogen with lymphocyte membrane receptors which serve to trigger lymphocyte activation. One consequence of this activation is the development of the lymphocyte's cytotoxic potential which is expressed *in vitro* by the release of a toxic lymphocyte product termed "lymphotoxin" (LT) (11–13). Lymphotoxin or "lymphotoxin-like" materials are released by lymphocytes activated under various conditions, i.e., when immune lymphocytes are cultured with sensitizing tissue (12, 14) or soluble antigens (15–18), when nonimmune lymphocytes are incubated with PHA or Poke weed mitogen (PWM) (13, 19, 20), and when nonimmune lymphocytes are incubated together in mixed cultures (21).

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This communication describes some of the physical and chemical properties of lymphotoxin secreted *in vitro* by PHA-stimulated mouse small lymphocytes. The characteristics of the mouse factor and previously reported human factor (22) are compared.

MATERIALS AND METHODS

Animals. Random bred Swiss-Webster and inbred C57B1/6 mice employed in these studies were obtained from Simonsen Laboratories, Inc., Gilroy, California.

Tissue culture cell lines and mitogenic agents. Established cell lines were obtained from Flow Laboratories, Los Angeles, California, and maintained in Eagle's minimal essential medium with 7% fetal bovine serum (MEM) as previously described (12). The cell lines used and their derivative tissues are as follows: HeLa—human carcinoma of the cervix; Ad-7—adenovirus transformed hamster cells; MBK—Moden bovine kidney; MA-111—neonate rabbit kidney; and L—mouse fibroblast. Phytohemagglutinin-P (PHA-P) was obtained from Difco Laboratories, Detroit, Michigan.

Preparation of lymphotoxin medium. The details of this technique have been described previously (12). Briefly, stock L cell monolayers (30×10^6 cells), established in 32-oz prescription bottles, were coated with PHA-P by incubation with 10 ml of MEH containing 160 μg PHA-P/ml. After 2 hr of incubation at 37° the medium was discarded and replaced with 50 ml of fresh MEM containing 150×10^6 purified Swiss-Webster of C57B1/6 splenic small lymphocytes (12). The cells were allowed to interact for 48–72 hr, at which time the media were collected, pooled, and cleared of cells and cell debris by centrifugation (1500 g for 30 min) and passage through a Millipore filter (0.45-μ pore size). All media were stored at −18° until used.

A number of experimental difficulties were encountered in the production of LT-containing media. One of the most troublesome was that the levels of LT in these media varied from one experiment to another. We found that PHA-P induced LT secretion, but PHA-M elicited only low levels of LT which, in many instances, were too low to be reproducibly detected. Moreover, additional studies revealed that the stimulating activity of PHA-P varied between lot numbers and many different batches were screened before selecting one that had maximum activity. Furthermore, as we found that PHA-P was unstable to storage in aqueous solution, it was stored in dry form according to the manufacturer's specifications and used immediately after solubilization. The highest and most consistent levels of LT were achieved by employing a serum-containing medium and by activating the lymphocytes on an L cell monolayer as opposed to activation with PHA alone. Under our conditions, serum-free media such as 199 and RMPI-1640 were unable to support the reproducible production of mouse lymphotoxin (MLT).

Preparation of control medium. Mouse L cells (2×10^8) were suspended in 10 ml of sterile distilled water at 4°. After 10 min of incubation, the cells were homogenized in a Potter-Elvehjem tissue grinder as previously described (12). Concurrently, suspensions of small lymphocytes were prepared from the spleens of nonimmune Swiss-Webster or C57B1/6 mice (12). Lymphocytes (2×10^9) were suspended in 10 ml of distilled water containing 5 mg of PHA-P. After 10 min of

incubation at 4° the lymphocytes were ruptured as described for L cells. The lymphocyte-PHA mixture was then combined with the L cell homogenate, and the total suspension was then added to 300 ml of conditioned MEM (collected from 48-hr cultures of L cells). The preparation was then centrifuged at 40,000 *g* for 30 min, and the supernatant was passed through a Millipore filter. After 48 hr of incubation at 37° the medium was stored at −19° until used.

Cytotoxicity assay: Monitoring protein synthesis as a measure of target cell viability. An aliquot of ^{14}C-labeled reconstituted protein hydrolysate, obtained from Schwarz Bioresearch, Van Nuys, California, was diluted in MEM labeling medium (12). The medium in each tube culture of target L cells to be labeled was drained to remove ^{12}C amino acids. Two milliliters of warm (37°) isotope-containing medium was added to each tube; the tubes were resealed and incubated at 37° for 20–45 min. After incubation the labeled cellular protein was extracted and quantitated as previously described (12).

MEM concentrate. In order to test low ionic strength solutions for cytotoxicity, they were reconstituted to physiologic salt and MEM nutritional levels by the addition of a 4 × MEM concentrate.

pH Studies. The pH of a series of tubes containing C57B1/6 MLT or control media was adjusted to different values by adding either 1 *M* HCL or 1 *M* NaOH. After 48 hr of incubation at 37° the media were dialyzed 18 hr against 1×10^{-3} *M* Tris–HCl, 1×10^{-4} *M* $MgCl_2$ (TM buffer), pH 7.6 at 4°. The samples were collected, reconstituted to MEM, and passed through Millipore filters onto L cell monolayers.

Prolonged dialysis of mouse lymphotoxin. Test and control MEM were placed in ¼-in. dialysis tubing and dialyzed at 4° against a 150-fold excess of TM buffer, pH 7.2. The dialysate was changed at 12 hr, 2, 5, 9, and 15 days. In addition, test and control MEM were placed in screw-capped test tubes with fragments of dialysis tubing, stored at 4°, and collected at the same time intervals as the dialyzing samples. Samples were collected at various time intervals and frozen at −18°. After all samples had been collected, the dialyzed samples were reconstituted with MEM concentrate, and the control media received the same volume of MEM. These media were then assayed for cytotoxicity on L cell monolayers.

Ammonium sulfate fractionation. Experimental and control media were subjected to stepwise ammonium sulfate fractionation. All steps were conducted at 4°. A flask containing 100 ml of MLT or control medium was placed in an ice bath atop a magnetic stirrer, and 38.5 g of granular ammonium sulfate (50% saturation) was gradually added. After the last aliquot was added, the solution was stirred for an additional 30 min before the precipitate was sedimented at 50,000 *g* for 20 min. The pellet was discarded, and 26.9 g of ammonium sulfate (to give 85% saturation) was added to the supernatant as described above. The precipitate was sedimented and resuspended to 6 ml with distilled H_2O. The sample was dialyzed against several changes of TM buffer, pH 7.2, for 24–30 hr and the volume adjusted to 8–10 ml, filter sterilized, and stored at −18° until used.

Phenol extraction. Test and control media were extracted twice with an equal volume of 0.15 *M* NaCl equilibrated phenol (Mallinckrodt, Los Angeles, Calif.) at 56° for 30 min. The aqueous phase was washed six times with ethyl ether and

dialyzed at 4° against TM buffer, pH 5.0. The samples were reconstituted, filter sterilized, and tested.

CsCl equilibrium density-gradient centrifugation. Preformed CsCl density gradients (from 3–50%) were prepared in 5-ml cellulose nitrate tubes. The samples were then centrifuged at 150,000 *g* for 36 hr at 4°. The tubes were punctured from the bottom and ten 33-drop fractions collected. The refractive index of each fraction was measured with a Bausch and Lomb refractometer and the density thus obtained from a standard curve. The volume of each fraction was adjusted to 1.5 ml with distilled water and dialyzed for 36–48 hr at 4° against several changes of TM buffer, pH 7.2. The samples were collected, supplemented with MEM concentrate, filtered, and tested on target cells.

Molecular sieve column chromatography. Sephadex G-100 slurry was poured into a Siliclad (Clay Adams)-coated glass column 1 cm in diameter to a final height of 38 cm. The gel was equilibrated for 48 hr with TM buffer, pH 8.6, containing 0.02 *M* NaCl at 4°. A 1.5 ml sample of ammonium sulfate fractionated MLT, containing 5×10^{-3} units of *E. coli* alkaline phosphatase (mol wt 80,000, Worthington Biochemical Corp.) and 0.05 ml of blue dextran solution (mol wt 2×10^6, Pharmacia Chemicals), was applied to the column. The flow rate was adjusted to 12 ml/hr, and 1.6 ml fractions were collected. Each fraction was assayed for alkaline phosphatase activity (23), and the blue dextran peak was located by absorption at 610 mμ. A 10 × ammonium sulfate concentrated slurry control sample was separated in an identical manner. Each fraction was tested for cytotoxicity on L cell monolayers.

RESULTS

Specificity of mouse lymphotoxin cytotoxicity. The following cell lines were established in tube cultures (2×10^5 cells/2 ml culture): MBK, AD-7, MA-111, HeLa, and L. Medium containing C57B1/6 MLT was serially diluted with MEM and incubated on duplicate cultures of the various test monolayers. After 48 hr of incubation at 37° the cultures were examined with the light microscope and pulse labeled with 0.5 μCi/ml of the ^{14}C amino acid mixture. These studies were repeated five times, and the results of a typical experiment are presented in Fig. 1 and indicate, with the exception of MBK, that all cell types tested were susceptible to undiluted MLT. However, upon dilution, the degree of cytolysis was variable between the different cell lines. Target cell synthesis was significantly elevated over controls when exposed to low levels of LT medium.

Heat stability. Aliquots of MLT and control media were placed in a 100° water bath and after various time intervals samples were removed and cooled to 4°. After the samples had been cleared of precipitate by centrifugation at 100,000 *g* for 2 hr, they were warmed to 37° and tested for cytotoxicity on L cell monolayers. After 48 hr of incubation the cultures were labeled for 20 min with 0.5 μCi/ml of the ^{14}C amino acid mixture. The results shown in Fig. 2 indicate that heated controls supported L cell growth while MLT was still active after 30 min of heating. These studies were repeated many times with essentially identical results. Also, control slurry maintained accelerated cell growth when compared with MEM, probably because of its higher protein concentration.

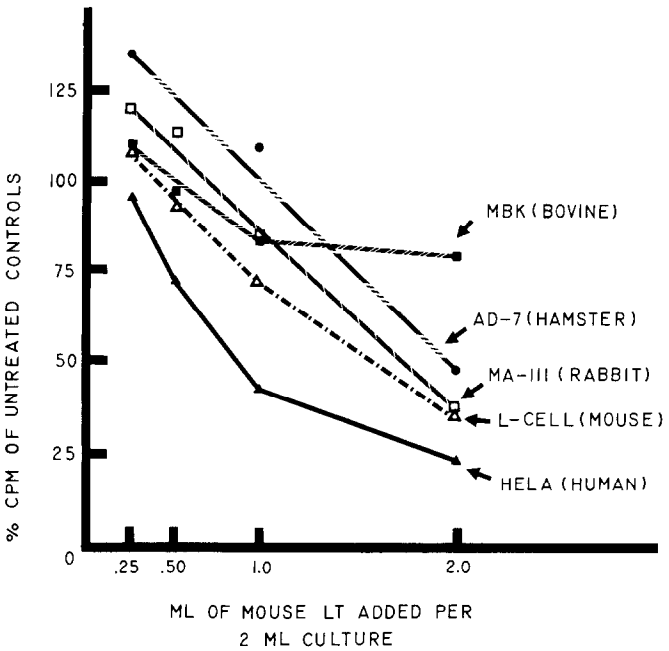

FIG. 1. Specificity of cytotoxicity. Duplicate cultures of the established lines indicated were treated with serial dilutions of C57B1/6 LT. After 48 hr incubation the cells were labeled with 0.5 μCi ^{14}C amino acids/ml for 30 min. Percent cpm of untreated controls equals (cpm of test cultures)/(cpm of control culture) $\times$ 100%.

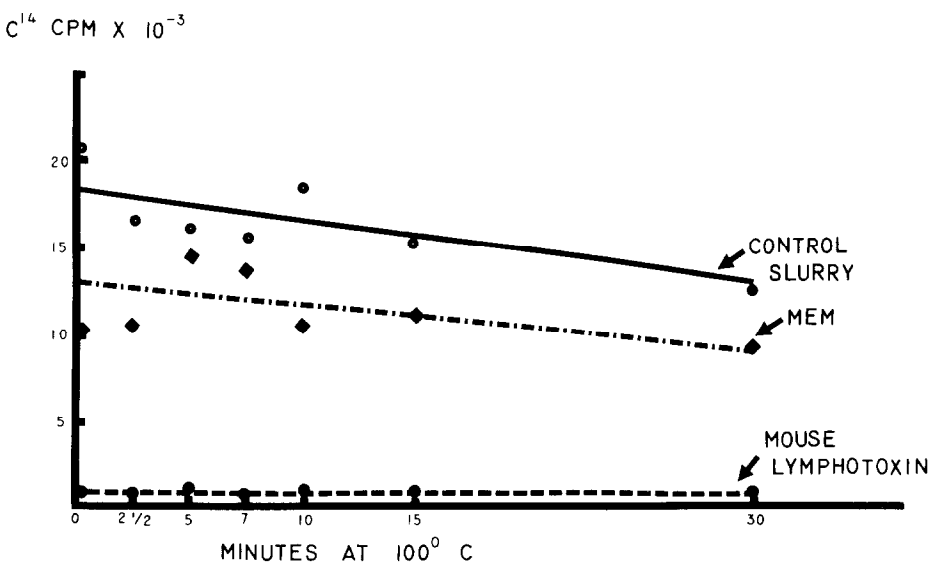

FIG. 2. Heat stability. Mouse LT, control slurry, and MEM were heated at 100° for the time intervals indicated, cooled, and assayed for cytotoxicity on duplicate monolayers of L cells. After 48 hr incubation the L cells were labeled with 0.5 μCi ^{14}C amino acids/ml for 20 min.

pH Stability. MLT and control media were adjusted to various pH values as described in Materials and Methods. After this treatment, duplicate samples from three separate experiments were tested for toxicity on L cells. Cell viability was measured after 48 hr incubation, the duplicate values were averaged, and the results of one of these experiments are presented in Fig. 3.

Stability to prolonged dialysis. Lymphotoxin and control media were dialyzed as described in Materials and Methods, and the media from duplicate experiments were tested for toxicity on L cell monolayers. After 60 hr incubation the cultures were labeled for 30 min with 0.25 μCi of ^{14}C amino acids/ml. The results in Fig. 4 indicate that MLT stored at 4° for 20 days retained activity while dialyzed MLT demonstrated a progressive loss of cytotoxicity. Periodic microscopic examination of these cultures revealed that L cells incubated with MLT subjected to prolonged dialysis showed characteristic vacuolation, described previously (12, 24), throughout the assay period. However, the cells continued to divide and incorporate high levels of ^{14}C amino acids. The level of this incorporation, however, was not as great as controls.

Ammonium sulfate fractionation. Lymphotoxin and control media were fractionated and concentrated by addition of various amounts of saturated ammonium sulfate solution. The final concentration of the different fractions obtained was adjusted to four times that of the starting media. The fractions were assayed for cytotoxicity on L cell monolayers, and after 48 hr of incubation the cultures were microscopically examined and labeled with 0.40 μCi of the ^{14}C amino acid mixture/ml for 25 min. The results are presented in Table 1. The major portion of toxic activity was recovered in the 60–80% fraction with some toxicity evident in the 35–60% fraction. A 50–85% fraction was subsequently employed to concentrate MLT for experimental use as outlined in the Materials and Methods.

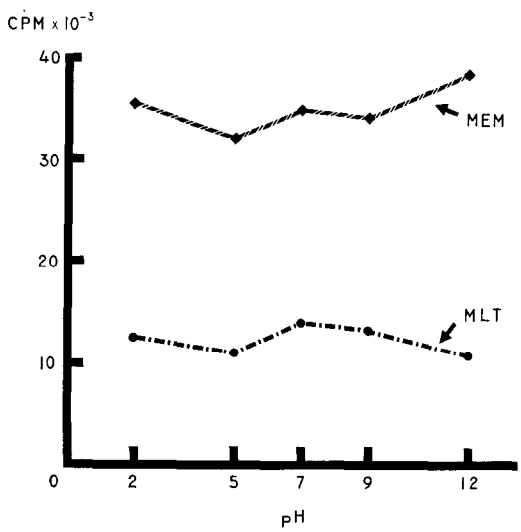

FIG. 3. pH Stability. Mouse LT and control MEM were adjusted to various pH values, incubated at 37° for 48 hr, neutralized as outlined in the text, and assayed on L cell monolayers. After 48 hr the L cells were pulse labeled with 0.5 μCi ^{14}C amino acids/ml for 40 min.

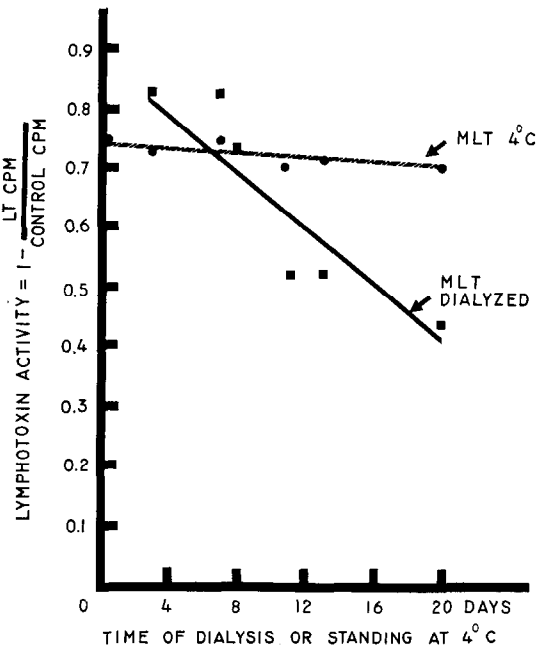

FIG. 4. Stability to prolonged dialysis. Mouse LT and control media were either stored or dialyzed at 4° for the time periods indicated and subsequently assayed for cytotoxicity on L cell monolayers. After 60 hr incubation the L cells were labeled with 0.25 μCi ^{14}C amino acids/ml for 30 min. The data have been normalized with respect to control by plotting lymphotoxin activity as 1 − (cpm incorporated into LT-treated cultures/cpm incorporated into control cultures).

Phenol extraction. Ammonium sulfate concentrated C57B1/6 MLT and control slurry were subjected to phenol extraction as described in Materials and Methods. The individual samples from three separate experiments were tested on L cell monolayers, and after 48 hr incubation they were assayed for viability. The results

TABLE 1

AMMONIUM SULFATE PRECIPITATION OF MLT[a]

Test medium	Ammonium sulfate concentration (% saturation)	cpm into target cell protein	% Cell killing
MLT	None	14,218	20
MEM	None	17,830	
MLT	0–35	14,405	2.5
MEM	0–35	14,842	
MLT	35–60	12,732	17.5
MEM	35–60	15,484	
MLT	60–80	9,757	49
MEM	60–80	18,998	

[a] Test and control media were subjected to stepwise ammonium sulfate fractionation. The precipitates were solubilized to a final concentration of 4× and assayed for cytotoxicity on target L cells, which were labeled after 48 hr with 0.40 μCi ^{14}C amino acids/ml for 25 min.

of a typical experiment are presented in Table 2 and indicate that phenol extraction inactivated cytotoxicity.

Buoyant density. Lymphotoxin and control media were subjected to CsCl equilibrium density-gradient centrifugation. A preformed linear gradient ranging from 3–50% CsCl was used, and fractions were collected and assayed for cytotoxicity on L cell monolayers. After 60 hr incubation, target cells were pulse labeled with 0.5 μCi/ml of the ^{14}C amino acid labeling medium for 45 min. From the results shown in Fig. 5 it is apparent that mouse LT banded at a buoyant density of 1.30 $\pm$0.04, a density corresponding to that of protein (25). These cultures were examined under the light microscope during the course of the experiment, and it was noted that after 12–24 hr incubation, fractions 4, 5, and 6 all showed L cell cytoplasmic vacuolation characteristic of MLT destruction (24). The L cell destruction in fraction 5 was progressive over the subsequent 36 hr until the monolayer was essentially destroyed. Cytoplasmic vacuolation was evident in tubes 4 and 6; however, the cells were able to divide and had the same protein synthetic activity as controls.

Sephadex column chromatograph. Sephadex G-100 was employed to determine the number of components responsible for mouse LT activity and to approximate molecular weight. The fractions collected were assayed for cytotoxicity in the usual manner. A typical result of one of these experiments is illustrated in Fig. 6, where target cell viability is plotted vs. fraction number; Ve/Vt equals elution volume/total volume (total volume was calculated using a phenol red marker, mol wt 394). Two marker peaks are shown, i.e., the excluded blue dextran was located in fraction 6, while the 80,000 mol wt alkaline phosphatase demonstrated peak activity in fraction 9. Monolayers that received control medium fractions supported good target cell growth, while the mouse LT elution profile showed a single peak of cytotoxicity with maximum activity in tube number 7. Thus, mouse LT appeared to be a single component with a molecular weight greater than 80,000 and less than 150,000, assuming that it is a globular protein.

DISCUSSION

An observation made consistently during these studies was that mouse lymphotoxin often lost activity when subjected to experimental manipulations. Therefore, high activity MLT was always employed and individual experiments were repeated many times. This loss of activity may be explained by several possi-

TABLE 2

THE EFFECT OF PHENOL EXTRACTION ON MLT CYTOTOXICITY [a]

Treatment	Sample	cpm ^{14}C amino acids incorporated into target cell protein
Phenol extraction	MLT	6,283
	Control slurry	5,834
Nonextracted controls	MLT	2,691
	Control slurry	6,807

[a] Target L cells were labeled after 48 hr incubation with 0.25 μCi ^{14}C amino acids/ml for 15 min.

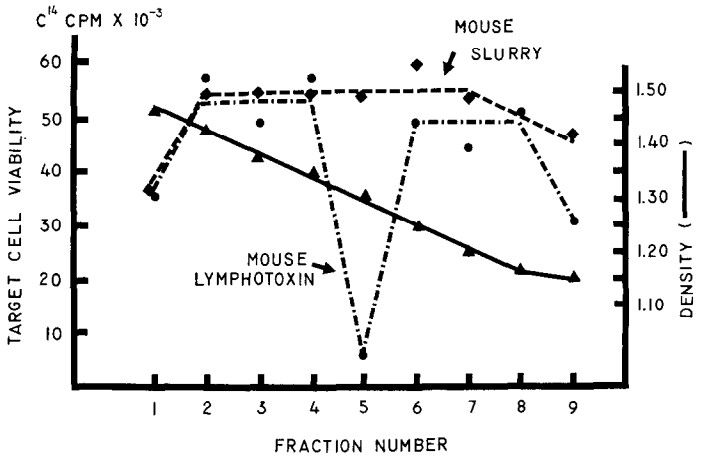

FIG. 5. CsCl equilibrium density-gradient centrifugation. Mouse LT and control media were subjected to CsCl density gradient centrifugation as outlined in the text. The various fractions were assayed for toxicity on L cell monolayers which were labeled after 60 hr incubation with 0.5 μCi ^{14}C amino acids/ml for 45 min.

bilities: (1) denaturation, (2) removal by adsorption to charged surfaces, or (3) inactivation by a mechanism that is currently unknown. The first two possibilities would appear unlikely because MLT is very stable to heat and extreme in pH; furthermore, it did not appear to adsorb to glass surfaces under the experimental conditions reported (see Fig. 4). Thus, it appears that unknown factors are necessary to stabilize the MLT protein, and they are currently under investigation.

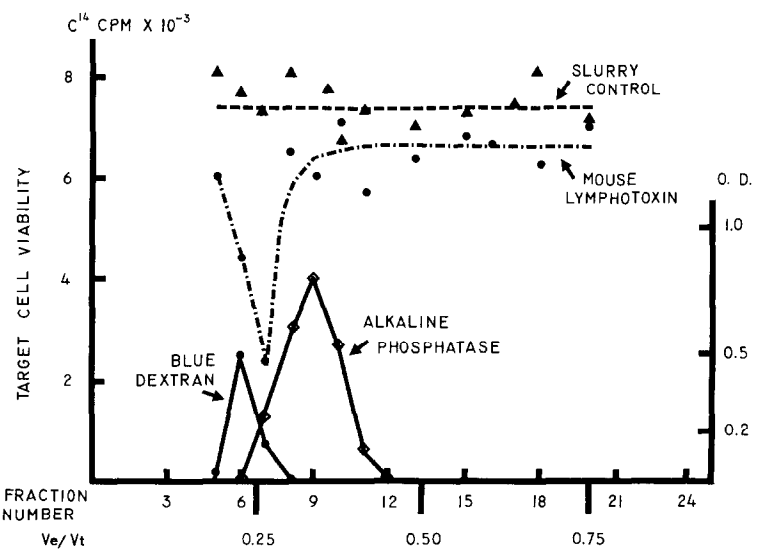

FIG. 6. Sephadex column chromatography. Ammonium sulfate-fractionated mouse LT and control slurry were subjected to Sephadex G-100 column chromatography, and the collected fractions were assayed for cytotoxicity on L cell monolayers as outlined in the text. After 48 hr incubation the target cells were labeled with 0.25 μCi ^{14}C amino acids/ml for 25 min. Ve/Vt equals the elution volume divided by the total volume.

It is possible that the presence of serum proteins and /or essential ions are required to stabilize its cytotoxic activity.

Mouse LT activity does not require heat-labile complement components nor is it associated with immunoglobulins because the toxic activity detected (1) remains after heating at 100° for 30 min, (2) is nonspecific, (3) is precipitated by 50–85% of ammonium sulfate saturation, and (4) possesses a molecular weight smaller than known immunoglobulins (26). The second point, however, should be qualified in that bovine kidney cells were somewhat resistant to MLT cytotoxicity, and preliminary studies have also indicated that kidney cells from a number of sources are refractory to human LT toxicity.

Data presented here and elsewhere (11) indicate that while undiluted MLT medium is cytotoxic for L cells, lower levels are stimulatory (see Fig. 1). One interpretation of this phenomenon would be that dilute MLT is stimulating target cells to repair and/or replace damaged membrane structures (24). The ultimate outcome of this interaction would then depend upon the number of LT-induced lesions vs. the efficiency of the cellular repair mechanism. This model is further substantiated by the observation that target cells treated with various inhibitors, such as dinitrophenol and cyclohexamide, are extremly sensitive to the destructive activity of LT (24).

The data presented in Table 3 indicate that MLT and HLT share certain properties, namely, identical buoyant densities and similar molecular weights. In contrast, they have different stabilities to dialysis, heat, and pH. They also differ in their behavior to ammonium sulfate fractionation. Additional studies (27) revealed that both mouse and human LT retained activity after incubation with the following enzyme preparations for 12 hr at 37°: 15 μg ribonuclease (RNase)/ml, 5 μg deoxyribonuclease (DNase)/ml, and 100 μg trypsin/ml. The insusceptibility of MLT to RNase and DNase in conjunction with its sensitivity to phenol extraction strongly suggests that its activity is not dependent upon nucleic acids. Although cytotoxicity was insusceptible to trypsin digestion under our conditions, MLT activity was clearly associated with protein as measured by CsCl equilibrium density centrifugation. The possibility still remains that cytotoxicity was due to a

TABLE 3

A COMPARISON OF THE PHYSICAL AND CHEMICAL PROPERTIES OF PHA-INDUCED MOUSE AND HUMAN LYMPHOTOXINS (27)

Experimental treatment	Mouse (27) LT	Human (22) LT
Heat	Stable to 100° for 15 min	Unstable to 80° for 15 min (27)
pH	Stable from pH 2–12	Stable from pH 5–9
Long-term dialysis	Progressive loss of activity	Stable during 21-day test
Ammonium sulfate fractionation	Precipitated at 50–85% of saturation	Precipitated at 0–40% of saturation
Phenol extraction	Sensitive	Sensitive
Sephadex chromatography	90–150,000 mol wt	80–90,000 mol wt
Buoyant density [a]	1.30 ± 0.04	1.30 ± 0.04

[a] Buoyant density values for macromolecules (23): Lipid, less than 1.0; protein, 1.33 ± 0.1; DNA, 1.71 ± 0.04; carbohydrate, 1.7–1.8; RNA, 2.0 ± 0.04.

small, nonproteinaceous material which associated with the protein fraction. However, this is unlikely because MLT would have to associate specifically with a single protein in the serum containing medium to yield consistently a complex of 90–150,000 mol wt.

Heise and Weiser (16) have recently demonstrated that the cytotoxic material(s) produced by guinea pig macrophages and lymphocytes appear to be somewhat larger than either HLT or MLT and are stable to heating for 30 min at 80° but not at 100°. This value is intermediate between the heat stabilities of human and mouse LT, and in conjunction with the data presented in this communication points to the fact that, although lymphotoxins produced by different species have similar biological activities, they have different physical and chemical properties.

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