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The Regulation of Lymphotoxin Release from Stimulated Human Lymphocyte Cultures: The Requirement for Continual Mitogen Stimulation

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Concanavalin A (Con A) activates nonimmune human lymphocytes *in vitro* to undergo transformation, DNA synthesis, and lymphotoxin (LT) secretion. LT secretion is inhibited (within minutes) when free and membrane-bound Con A are removed by washing or incubation with the competitive inhibitor, α-methyl mannoside. LT secretion can be reinitiated by addition of fresh Con A. While LT can be rapidly regulated, blast transformation and cellular DNA synthesis are under less restrictive control. Although they appear to be related, LT secretion and lymphocyte transformation seem to be regulated by independent control mechanisms. These studies indicate that recognition and contact of lymphocyte membrane sites initiate as well as regulate the efferent or destructive phase of cell-mediated immune (CMI) reactions. A model of how lymphocytes could employ LT in specific and nonspecific cytodestructive CMI reactions is presented.

INTRODUCTION

It has been established that lymphocytes from humans and experimental animals cause *in vitro* destruction of target cells against which they have been previously sensitized (1–4). The primary step in this destructive reaction appears to be contact between the aggressor lymphocyte and the target cell (5, 6). Contact induces the process of lymphocyte activation and the mechanism(s) which cause specific destruction of the donor cells. Investigators have reported this mechanism to be highly specific (1–3), while others have suggested that low levels of nonspecific destruction may occur to bystander cells of other genotypes (7, 8). In contrast, lymphocytes from donors immune to soluble antigens cause a nonspecific destruction of target cells *in vitro* when cultured in the presence of the specific stimulating antigen (9–11). It is also possible to induce a nonspecific cytolytic reaction by incubating nonimmune lymphoid cells with target cells in the presence of various mitogens; agents known to cause cellular aggregation and lymphocyte transformation (12–14). The above models indicate that the cytotoxic reaction is multifaceted, composed of induction and effector steps which may be specific or nonspecific, depending upon the particular system being studied.

Human lymphocytes, following treatment with the plant lectins, phytohemagglutinin or concanavalin A, or with antigens, undergo blastogenesis and synthesize increased levels of DNA, RNA and protein, as well as secrete into culture medium

a number of soluble factors termed collectively "lymphokines" (15). These substances are known to have a wide range of effects upon other cells *in vitro* (15–16). One of these molecules has been termed "lymphotoxin" (LT), an apparently nonspecific cell toxin, having a diverse range of cytolytic activity on different cell types *in vitro* (17, 18). Lymphotoxin has been detected in culture supernatants from lymphocytes activated by mitogens, antigens, and in mixed leukocyte reactions (19, 20). In CMI reactions, a strict control would have to be placed upon the action of a soluble nonspecific cell toxin, since indsicriminate synthesis and secretion would be disadvantageous to the organism. We have found that one level of control for LT appears to come through functional restriction initiated by its binding to target cell membranes (21). This report determines the presence of another and more important level of control; namely, that continual membrane stimulation is required for LT secretion by activated lymphocytes. This finding suggests an exquisitely sensitive method for the modulation of effector molecule release that can be turned on and off independently of morphologic transformation and DNA synthesis. A model of how lymphocytes could employ LT to mediate cytodestructive reactions is presented.

MATERIALS AND METHODS

L Cells

An exquisitely sensitive strain of mouse L cells isolated in our laboratory (alpha subline) was used in this investigation. The degree of sensitivity to direct lymphocyte cytolysis and lymphotoxin-mediated cytolysis and other characteristics of this cell line have recently been reported in detail (22). Briefly, the cells were maintained as monolayers in Minimum Essential Medium with Hanks' salts, supplemented with 5% newborn calf serum (Microbiological Associates, Bethesda, MD), penicillin (100 U/ml), and streptomycin (100 μg/ml) (MEMS). Cells were maintained at low density in 8 or 16 oz prescription bottles and passed biweekly.

Lymphocyte Cultures

Suspensions of human small lymphocytes were obtained from surgically removed adenoids of normal children, as previously described (23). Lymphocyte suspensions were adjusted to a concentration of 3×10^6 viable cells/ml in MEMS containing 2.5 μg/ml fungizone. 2.0-ml aliquots were cultured in 16×125-mm screw-capped tubes and gassed with 5% CO_2 in air. Activation of the lymphocyte cultures was effected by addition of concanavalin A (Con A, Sigma, St. Louis, MO, Lot. No. 73C-5030), or phytohemagglutinin (PHA-P) (Difco Laboratories, Detroit, MI, Lot No. 573353). Optimum concentrations of both mitogens giving good stimulation of DNA synthesis and high levels of LT activity was determined to be approximately 20–40 μg/ml. Following incubation at 37°C, for various time intervals, supernatants were collected by centrifugation at 300*g* for 10 min, followed by passage through .45 micron Millipore filters. The cell pellets were washed by the addition of 5 volumes of phosphate-buffered saline (PBS, 0.15 *M* NaCl, 0.015 *M* PO_4 buffer, pH 7.2), or PBS containing 5×10^{-2} *M* α-methyl-mannoside (MAM, Sigma, St. Louis, MO).

Lymphotoxin Assay System

Lymphotoxin (LT) was assayed in culture supernatants by determining the percent survival of mitomycin C-treated target cells. This procedure has recently been described in detail (24). Briefly, L cells were cultured as monolayers in tubes at a concentration of 10^5 cells/ml in the presence of 0.5 μg/ml mitomycin C (Sigma, St. Louis, MO). Following overnight incubation at 37°C, the supernatants were decanted and serial dilutions of LT-containing medium or control medium in MEMS added. The cells were then incubated for 24 hr, and the remaining adherent cells removed by trypsin treatment and enumerated by Coulter Counter evaluation. One unit of LT activity is defined as the amount of LT necessary to effect a 50% reduction in the number of viable cells following a 24-hr incubation.

Determination of DNA Synthesis

Washed cell pellets from both stimulated and control human lymphocyte cultures were suspended in 2.0 ml of MEMS containing 1 μCi/ml of tritiated thymidine (^{3}H-Thy, 6 Ci/m*M*, Schwartz, Orangeburg, NY), and incubated for 4 hr at 37°C in an atmosphere of 5% CO_2 in air. Triplicate cell cultures were harvested by centrifugation at 500*g* for 10 min. The pellet was washed with 10 ml of PBS, followed by precipitation of the DNA with 10% trichloroacetic acid (TCA). The precipitates were collected onto glass fiber filters (Whatman GF/A), placed into scintillation vials and 0.5 ml of NCS (Amersham/Searle, Arlington Heights, IL) added. Following digestion for 1–2 hr, 10 ml of toluene scintillation fluid (0.4% omnifluor in toluene) was added, and radioactivity determined on a Beckman LS-100 scintillation counter.

RESULTS

Concanavalin A Dose Response Curve—The Relationship Between ^{3}H-Thymidine Incorporation and Level of LT Secreted

Lymphocytes were cultured for 72 hr in the presence of various concentrations of Con A. The cell cultures were then centrifuged, supernatants collected, and 2 ml of fresh medium containing 2 μCi of ^{3}H-Thy added. Following a 3-hr pulse, the incorporation of radioactivity into TCA insoluble material was determined. Lymphotoxin levels on the 72-hr supernatants were assayed as described in Materials and Methods. The results (Table 1) indicate that 20 μg of Con A/ml produced optimum DNA synthesis under our conditions, whereas 40 μg/ml was optimal for the amount of LT secreted. We employed 20 μg/ml of Con A routinely during this investigation for lymphocyte stimulation, since this concentration was near the optimum for both DNA synthesis and LT activity.

Concanavalin A Stimulation of Lymphocytes—Kinetics of Lymphotoxin Release

The kinetics of LT release from stimulated lymphocytes was determined on supernatant medium collected at daily intervals from adenoid lymphocytes cultured in the presence and absence of Con A. All supernatants were stored frozen at −20°C, then all assayed at the same time. Figure 1 illustrates the LT activity obtained from culture supernatants between Day 1 and Day 6, following stimulation. The results clearly indicate that high levels of LT are secreted by 48 hr after stimu-

TABLE 1

CONCANAVALIN A DOSE RESPONSE CURVE ON HUMAN LYMPHOCYTE DNA AND LT SYNTHESIS IN VITRO

Con-A (μg/ml)	^{3}H-Thymidine incorporated (cpm/culture)	LT activity (units/ml)
0	550[a]	<10[a]
5	87,401	125
10	134,687	700
20	188,021	2,800
40	36,021	3,400
80	3,312	730

[a] Mean of triplicate cultures. Representative data from experiment 2 of three experiments. Deviations from the mean were consistently less than 12%.

lation, and LT activity continued to increase until Day 4, when it leveled off. Since high activity was detected in supernatants at 48-hr poststimulation, this incubation period was employed as a standard prestimulation time for all the following experiments.

Mitogen Stimulation of Human Lymphocytes—The Selective Inhibition of Con-A Stimulated Lymphocytes by α-Methyl Mannoside

α-methyl mannoside (MAM) is a competitive inhibitor of Con A, and binds selectively to the active sites on the Con A molecule. It has also been determined that the binding of Con A to human lymphocytes is dissociated by the addition of MAM (25). Experiments were therefore conducted to determine the extent and specificity of MAM inhibition on Con A-stimulated lymphocyte cultures. Adenoid lymphocytes were cultured in MEMS, as well as in MEMS containing 5×10^{-2} *M*

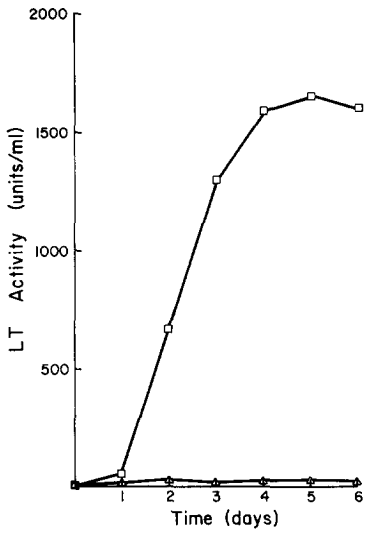

FIG. 1. The kinetics of LT release from CON-A stimulated lymphocytes. (□) LT activity from cultures stimulated with 20 μg/ml CON-A, (△) unstimulated cultures. Data is the mean activity of triplicate cultures in Units/ml.

TABLE 2

THE EFFECT OF α-METHYL MANNOSIDE ON PHA-P AND CON-A STIMULATED HUMAN LYMPHOCYTE CULTURES

Culture conditions	^{3}H-thymidine incorporated (cpm/culture)	LT activity (units/ml)
PHA	246,143[a]	6,000[a]
PHA and MAM	296,460	5,750
Con-A	138,746	2,500
Con-A and MAM	4,171	<10
MEMS control	3,178	<10

[a] Mean activities from triplicate cultures. Representative data from Experiment 1 of two experiments. Deviations from the mean were consistntly less than 13.1%.

MAM. The 2-ml cultures were stimulated by the addition of either 20 μg/ml of PHA-P, or 20 μg/ml Con A. Following 3 days of incubation at 37°C, 2 μCi of ^{3}H-Thy was added for 4 hr, followed by collection of the supernatants for LT assay and TCA precipitation of the cell pellet to determine the amount of incorporated radioactivity into TCA precipitable material. The results (Table 2) indicate that Con A did not activate the lymphocytes when incubated in the presence of MAM. Lymphocytes stimulated in the presence and absence of MAM, as well as the Con A-stimulated cultures in the absence of MAM, underwent normal blast transformation and released high levels of LT into the medium. These results indicate that the MAM selectively inhibited the mitogenic influence of the Con A on the lymphocytes and had no effect upon lymphocyte stimulation by an unrelated mitogen.

The Requirement of Continual Stimulation to Maintain LT Secretion by Lymphocytes

Experiments were done to determine whether LT secretion by stimulated lymphocytes *in vitro* required continual mitogen stimulation. Human lymphocytes were incubated with Con A for 48 hr to initiate a high rate of LT secretion. The supernatants were then collected and the activated cells washed with PBS and resuspended in fresh medium containing Con A or fresh medium alone. An additional set of prestimulated cultures were centrifuged, washed with PBS containing 5×10^{-2} *M* MAM, and resuspended in MEMS containing the competitive inhibitor. Control cultures were incubated for 48 hr in medium alone, washed with PBS, and the cell pellet resuspended in fresh medium. Following an additional 48 hr of incubation at 37°C, supernatants were collected and LT titers determined. Figure 2 indicates that the Con A restimulated cultures maintained a high rate of LT synthesis (greater than 1,200 U/ml), however, lymphocyte cultures washed free of soluble mitogen, exhibited a 84% decrease in the amount of LT activity released during the 48-hr incubation period (200 U/ml). Washing the cell pellet with PBS containing the competitive inhibitor, followed by incubation in MEMS plus MAM further reduced the activity of LT secreted during the 48-hr incubation period (45 U/ml). This was greater than 95% reduction in LT activity, as compared to the amounts secreted with constant mitogen stimulation. Control cultures exhibited only minimal LT activity (less than 10 U/ml). A secondary treatment of

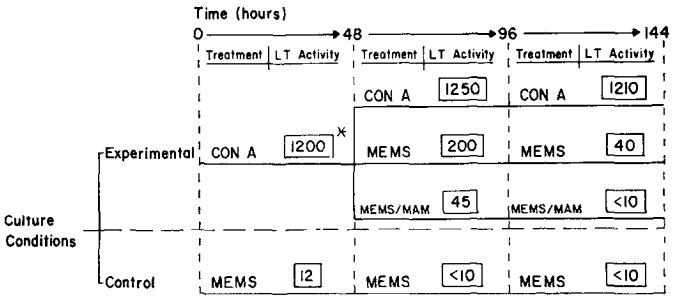

FIG. 2. The protocol used for the inhibition of CON-A stimulated lymphocytes by MAM. * Values in boxes are the mean LT activities of triplicate cultures in Units/ml.

cultures at hour 96 by washing, followed by a third 48-hr incubation, caused a further reduction in LT activity under conditions where mitogen had been removed. Both the cultures incubated without mitogen, and the cultures incubated with added MAM exhibited an additional fivefold drop in activity, while the Con A restimulated cultures maintained an LT activity of approximately 1200 U/ml.

Restimulation of MAM Inhibited Cultures

Experiments were conducted to determine whether MAM inhibited lymphocytes could be restimulated into LT-secreting activity, or if they were irreversibly inactivated by this treatment. Lymphocytes were stimulated with Con A for 48 hr, washed, and fresh medium or medium containing MAM or Con A was added for an additional 48 hr. The cells were then centrifuged, supernatants collected, assayed for LT, and the lymphocytes restimulated by the addition of fresh Con A. Following a 48-hr incubation period, the cell supernatants were collected and assayed for LT activity. Results depicted in Fig. 3 indicated that at 48 hr, the supernatants from Con A stimulated cells had an LT activity of approximately 1,260 U/ml. Addition of MAM dropped the activity of these cultures to approximately 50 U/ml and the readdition of Con A to this culture system caused a restimulation of the lymphocytes to an activity of 980 U/ml. This experiment indicates that the LT secreting population of lymphocytes responding to stimulation with Con A apparently are not irreversibly shut down by the addition of MAM, since LT secretion was reinitiated following secondary stimulation of the cells. Control values consistently gave less than 10 U of activity per ml.

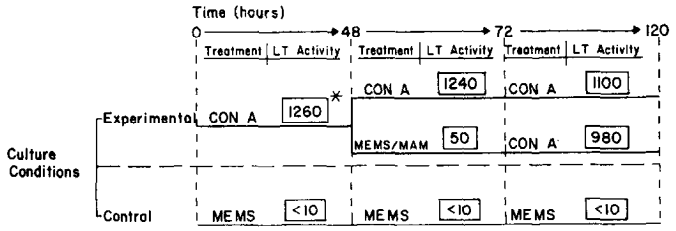

FIG. 3. The protocol used for the mitogen restimulation of MAM-inhibited lymphocytes. * Values in boxes are the mean LT activities of triplicate cultures in Units/ml.

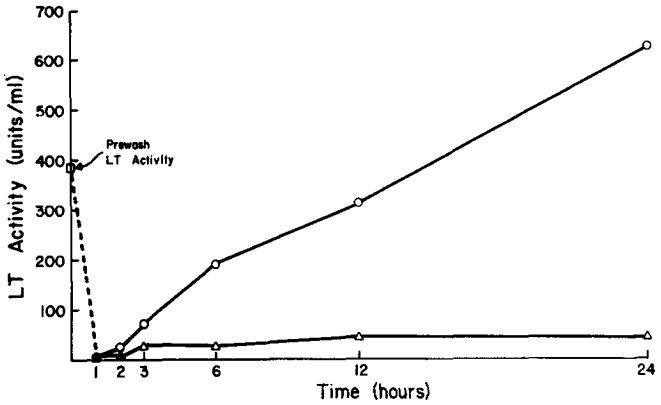

FIG. 4. Kinetics of LT release from CON-A-stimulated lymphocytes following the restimulation with fresh CON-A (○) or following addition of MAM (△). Data is the mean of triplicate cultures in Units of LT/ml.

The Kinetics of LT Inhibition by MAM Addition to Con-A Stimulated Lymphocyte Cultures

To determine the length of time, following addition of MAM to Con A-stimulated cultures, necessary before complete shutdown of LT secretion is effected, the following experiments were conducted: Lymphocyte cultures were first stimulated by incubation for 48 hr with Con A. The cell cultures were then centrifuged and supernatants were collected. The cell pellets were washed and resuspended in fresh medium containing Con A or in medium containing the competitive inhibitor, MAM. At 1, 2, 3, 6, 12, and 24 hr, supernatants were collected and assayed for LT activity. The results (Fig. 4) indicate that high levels of LT are secreted in the medium, following restimulation of lymphocytes with fresh Con A. In fact, the level reached during this 24-hr period was greater than that reached during the primary 48-hr prestimulation period. Observable LT activity in these cultures was detected by 2 hr. Lymphocyte cultures were rapidly inhibited from further LT secretion by the addition of MAM to the culture system. Differences from the positive controls were observed 2 hr following the addition of the MAM. Background cytotoxic activity from unstimulated lymphocyte cultures was always below 10 U of activity per ml.

The Relationship Between the Regulation of DNA Synthesis and the Regulation of LT Synthesis by Stimulated Lymphocytes

Experiments were performed to determine the temporal relationship between the inhibition of LT secretion and the inhibition of ^{3}H-Thy uptake into MAM blocked Con A-stimulated lymphocyte cultures. Lymphocyte cultures were stimulated by incubation with Con A for 48 hr. LT activity in the supernatants of these cultures was approximately 1240 U/ml. Parallel cultures were treated with Con A in fresh medium, fresh medium alone, or fresh medium containing MAM. Following an additional 24 hr incubation, 2 μCi of ^{3}H-Thy was added for 4 hr. The supernatants were then collected and tested for LT activity, and the cell pellets processed to determine the level of incorporated radioactivity. The results, outlined in Table 3, indicate increased levels of incorporated ^{3}H-Thy were maintained for 24 hr fol-

TABLE 3

THE RELATIONSHIP BETWEEN ^{3}H-THYMIDINE INCORPORATION AND LT SECRETION IN STIMULATED LYMPHOYTE CULTURES

	Lymphocyte culture treatment	^{3}H-thymidine incorporated[a] (cpm/culture)	LT activity[a] (units/ml)
Experimental—48 hr Con-A prestimulation	Con-A/MEMS	23,311	1,250
	MEMS	42,827	240
	MAM/MEMS	30,179	40
Control—MEMS pretreatment	MEMS	354	<10

[a] Mean activities from triplicate cultures. Representative date from experiment 1 of two experiments. Deviations from the mean were consistently less than 11.8%.

lowing the addition of MAM to the Con A-stimulated cultures. The LT activity at this time, however, is completely inhibited. Further experiments indicated that inhibition of DNA synthesis was effected 48 hr after the addition of the competitive inhibitor. These results suggest that LT secretion is under a more sensitive and responsive control system than that which regulates DNA synthesis in human lymphocyte cultures.

DISCUSSION

These results indicate that secretion of lymphotoxin into culture supernatants by mitogen stimulated lymphocytes appears to be dependent upon the continual presence of the stimulating mitogen. In addition, this regulatory system appears to be both rapid and exquisitely sensitive. Washing of Con A-stimulated lymphocytes with PBS to remove the majority of the free mitogen was effective in reducing the amount of LT secreted to 20% of the restimulated controls during a subsequent 48-hr incubation period. Addition of MAM into the culture medium increased this shutdown of LT secretion to less than 4% of the controls. This inhibition of LT secretion caused by the addition of MAM was not due to nonspecific cytotoxicity, since MAM was determined to have no effect on either lymphocyte function or LT activity. PHA-P stimulated lymphocytes, in the presence of MAM, underwent normal blastogenesis and secreted high levels of LT. In addition, the presence of MAM in the supernatant medium had no effect on the target cell sensitivity to LT. It was also determined that LT secretion could be reinitiated in MAM-inhibited lymphocyte cultures by removal of the inhibitor and restimulation of the cells with fresh mitogen. Kinetic evaluation of LT release by MAM-inhibited lymphocyte cultures determined that almost total shutdown of LT secretion takes place within minutes following inhibitor addition. The very low level of cytotoxic activity observed may relate to the time required to effect a complete dissociation of Con A from the lymphocyte membrane. The rapidity of inhibition suggests that the actual control step is at a site on the lymphocyte membrane surface regulating LT secretion. However, this does not exclude the possibility that the actual control step directly stops LT synthesis.

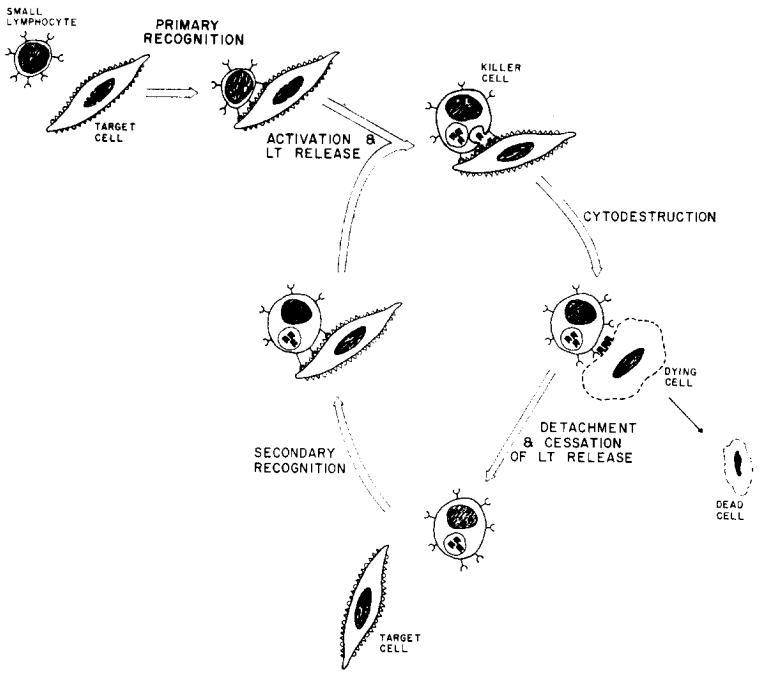

FIG. 5. Proposed model for the role and regulation of lymphotoxin in CMI cytodestructive reactions.

Recent reports indicate that restimulation of lymphocyte progeny with antigens or mitogens is necessary for continued proliferation (26, 27). The regulatory mechanism controlling this system, does not appear to be immediate, however, since maintenance of DNA synthesis through at least one round can take place following complete mitogen removal (25). Our results confirm these findings. Stimulation of human lymphocytes for 24 to 48 hr followed by mitogen removal failed to reduce the level of incorporated thymidine tested up to 24 hr later, while LT activity had been almost totally shutdown. This data suggests that the mechanism controlling LT release may be turned on or turned off irrespective of the lymphocytes stage of differentiation.

The requirement for constant stimulation to maintain LT release may have broad implications into the mechanism of how lymphocytes cause cytodestruction *in vitro*. The current controversy in this field centers around whether contact between lymphocytes and target cells alone is sufficient for cytotoxicity or whether binding induces the lymphocyte into secreting a cytotoxic effector molecule. Numerous reports have recently determined that in order for immune cytolysis to occur, cellular events following contact are required. The addition of agents known to affect secretory processes appears to have a pronounced effect upon the lymphocytes' cytodestructive ability (28–31). Increasing the intracellular levels of cyclic AMP (28, 29), as well as the addition of cytochalasin B (31), greatly reduces the cytolytic activity of immune lymphocytes. Other investigations by Möller *et al.* (32) and Ginsburg *et al.* (33), on mitogen-induced lymphocyte-mediated cytotoxicity, have independently determined that Con A pretreated lymphocytes failed to cause cell cytolysis following removal of the mitogen. This inhibition took place even though the lymphocytes were irreversibly activated into DNA synthesis, were

morphologically transformed, and made contact with the target cells. Cytotoxic activity was, however, only reestablished by addition of fresh Con A to the system. They concluded that contact alone was not sufficient for expression of cytotoxicity, and that mitogen-mediated binding was required. Another equally plausible explanation of these data is that they inhibited LT release in their lymphocyte cultures by Con A removal, and that addition of the fresh Con A reinitiated LT synthesis.

Figure 5 depicts our concept of the involvement of LT and the regulatory processes controlling cytodestructive CMI reactions. The lymphocyte, having been activated into effector function by intimate contact with target cell membrane antigens, is induced into LT synthesis. The LT, upon secretion, binds to the target cell membrane, where it is functionally restricted, and effects cell cytolysis. Target cell membrane disruption, recognition unit denaturation or physical dislodgment promotes the lymphocytes' release, followed immediately by shutdown of further LT secretion. This inhibits an indiscriminate wandering of an LT secreting lymphocyte and therefore regulates and prevents nonspecific destruction. Subsequent recognition and attachment to other target cells reinitiates the entire cytodestructive process. This could allow a single immune lymphocyte to cause destruction of many target cells sequentially, and the degree of lymphocyte induced cytodestruction would be directly related to the amount of LT released and the target cells sensitivity to LT action. We believe that the present findings may provide insight into the mechanism(s) controlling lymphocyte effector function in CMI reactions.

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