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The Human LT System

VII. Release of Soluble Forms and Delivery to Target L-929 Cells by Lectin-Preactivated Human Lymphocytes in the Absence of Protein Synthesis and Secretory Processes

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We have investigated the effects of inhibitors of cellular protein synthesis (emetine, cycloheximide) and secretion (colchicine, cytochalasin B) on the capacity of primary or secondary lectin-activated human lymphocytes to release LT molecules or to cause lectin-induced destruction (LICC) of murine L-929 cells in vitro. Our findings reveal: (a) agents which inhibit protein synthesis or secretion block the release of LT activity into the supernatant and LICC when primary lectin-stimulated human adenoid lymphocytes are employed as effector cells; (b) these same agents are ineffective at blocking LT release or LICC when 3- or 5-day lectin-prestimulated lymphocytes are employed; and (c) anti-human α-LT serum blocks LICC of L-929 cells mediated by primary or secondary lectin-activated human lymphocytes. The difference in participation of effector cellular processes in LICC between primary and secondary lectin-stimulated cells correlates with the findings that preactivated lymphoid cells possess high levels of preformed intracellular, as well as membrane associated, LT molecules, and that release of these materials into the supernatant or delivery to the target cell can occur independently of active protein biosynthesis or classical secretory systems.

INTRODUCTION

It is becoming evident that human lymphotoxins are a heterogeneous but interrelated family of cell-lytic proteins that are released into the soluble phase by activated lymphoid cells in vitro (1–6). Recent biochemical and immunologic data indicate these materials form a system of subunits (6–8). Certain smaller forms (α) can be dissociated from high MW complexes (CxS) (8). The latter can be functionally associated with antigen-binding receptor(s) (8, 9). Functional studies of various human LT MW classes indicate that, with the exception of the murine L-929 cell, the smaller MW forms are only weakly lytic, whereas Cx forms can cause rapid 51Cr release from a variety of cell types in vitro. In addition, alloimmune murine and human lymphoid cells can release Cx forms associated with target cell-specific receptors which can cause rapid “specific” target cell lysis (10, 11). Lymphocytes can be activated to release LT molecules in vitro by a wide spectrum of treatments (12). Studies with reversible inhibitors of protein synthesis revealed

1 This research was supported by Grant AI-09460, from the Institute of Allergy and Infectious Diseases, NIH, and Grant 1882, from the Rheumatic Diseases Research Foundation.
that primary lectin-activated nonimmune human lymphocytes required cellular protein synthesis for LT release in vitro (13). This result has been used by several investigators as one argument to negate the role of these molecules in murine T-lymphocyte killing, since lysis can occur in the apparent absence of effector cell protein synthesis (14, 15). However, relatively recent findings indicate that lectin-activated human lymphoid cells accumulate intracellular LT pools and can very rapidly release LT into the supernatant upon restimulation with lectin (2, 16). Moreover, lectin-activated human lymphoid cells, including T cells, can express α-LT molecules on their plasma membranes (17). These findings suggested there may be differences in the cellular processes required for LT release between lymphoid cells, depending upon their state of activation. The present studies confirm this hypothesis and reveal that once lymphoid cells are activated, LT release can occur in the complete absence of protein synthesis and low levels of LT, perhaps membrane-associated forms, can be released in the absence of active membrane secretion. LICC of L-929 target cells also follows a similar pattern.

MATERIALS AND METHODS

I. Target Cells and Culture Medium

Stock cultures of a sensitive strain of mouse L-929 fibroblasts (α-L-929) were used as target cells (11). These cells were maintained in 32-oz prescription bottles in 95% air, 5% CO₂, and passed biweekly. Culture medium consisted of minimal essential medium with Hanks salts, supplemented with 3% heat-inactivated (56°C, 60 min) fetal calf serum (Microbiological Associates, Bethesda, Md.), 0.2 μg/ml glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) (MEMS).

II. Lymphocyte Cultures

Single-cell suspensions of human lymphocytes from tonsils or adenoids were obtained within 6–8 hr of surgical removal from normal children, as previously described (18). Lymphocyte suspensions were adjusted to 4 x 10⁶ viable cells/ml in 32-oz prescription bottles, using MEMS, and incubated for 5–6 days in 95% air, 5% CO₂, at 37°C. Activation of the lymphocytes was effected by the addition of phytohemagglutinin-P (PHA-P, Difco Laboratories, Detroit, Mich.) at 20 μg/ml, or concanavalin A (Con A, Sigma, St. Louis, Mo.) at 5 μg/ml. Lymphocytes to be treated with colchicine, emetine, or cycloheximide (all obtained from Sigma) were incubated with the drug in MEMS at 37°C for given amounts of time indicated in the text. The cells were then washed with MEMS two to three times using 50 ml for each wash. When employed as effector cells, PHA-activated lymphocytes were pipetted vigorously to break up clumps and washed two times with MEMS before use. To those cultures, activated with Con A, α-methyl-D-mannoside (α-MAM, Sigma) was added to 5 mM, and the cells were vigorously pipetted, then washed two times (using 40 ml each) in MEMS plus 5 mM α-MAM to remove all residual cell bound Con A. Lymphocyte viability was determined by dye exclusion microscopic observation of cells suspended in 0.1% eosin Y solution.

III. Lymphotoxin Assay

The details of these methods have been reported previously (19). Briefly, 10⁵ L-929 target cells in 1.0 ml were established in 13 × 150-mm screw-capped tubes in
MEMS containing 0.5 µg/ml mitomycin C. After 24 hr incubation at 37°C, the medium was discarded, the monolayers were washed with PBS, and the remaining adherent cells were trypsinized and enumerated in a Model F Coulter Counter. Units of LT activity/milliliter of a given supernatant are obtained by determining the reciprocal of the dilution killing 50% of the target L cells.

IV. Lectin-Induced Lymphocyte-Mediated Cytotoxicity (LICC)

Target L cells (10⁵ cells in 1.0 ml MEMS containing 0.6 µg/ml mitomycin C) were established 24 hr prior to use, as previously described (20). The medium was discarded, the monolayers were washed with PBS, and to each tube 1 ml of a cell suspension containing a predetermined number of lymphocytes was added to give lymphocyte:target cell (Ly:TGC) ratios as indicated in the text. Concanavalin A was then added to each tube at 5 µg/ml or Con A plus 5 mM MAM was added as a control. The tubes were then incubated at 37°C for 15–24 hr, after which they were vigorously shaken, and the dead cells which had detached from the monolayers poured off in the supernatant. The remaining viable L cells were removed and counted in a manner identical to that used in the LT assay.

V. Production and Use of Anti-LT Serum

The methods for obtaining and testing specific antisera have been reported previously (7, 21). Briefly, New Zealand white rabbits were immunized with rechromatographed Sephadex fractions containing all subclasses of the 70,000–90,000 MW α class of human lymphotoxins but free of all other classes (B-series animals) suspended in complete Freund's adjuvant (CFA) (anti-α-LT). This antiserum or normal rabbit serum (NRS) was collected, heat inactivated (56°C, 45 min), and absorbed on unactivated human lymphocytes (10⁶/ml serum) and L cells (10⁶/ml) for 1–2 hr at 4°C; the cells were removed by centrifugation, and the serum was filter sterilized before use.

VI. Lymphocyte Supernatants

Supernatants from activated lymphocytes (SAL) were collected by centrifugation at 400g for 10 min, followed by filtration through a 0.45-µm Millipore filter. These supernatants, if stored, were stored at −20°C until used. SAL obtained from cultures employing inhibitors were dialyzed for 24 hr at 4°C against 1000 vol of PBS.

VII. Whole Cell Disruption

Homogenates of whole cells were obtained as described previously (2). Briefly, lymphocytes were washed with MEM containing 1% fetal calf serum and resuspended in 3 to 4 ml of fresh MEM. They were then sonicated for 6 min at 25°C, using a Bransonic 32 sonicator. Microscopic observation verified that >99% lysis had occurred. The homogenates were then centrifuged, 20,000g, 15 min, and the supernatants sterilized by passage through a 0.22-µm Millipore filter.

VIII. Determination of Lymphocyte Protein and DNA Synthesis

Cultures of human adenoid lymphocytes were established in 13 × 150-mm plastic screw-capped tubes (Falcon, Oxnard, Calif.) at 4 × 10⁶/ml (2 ml/tube) in MEMS and incubated for 2 hr in the presence of various concentrations of cycloheximide or
emetine. The lymphocytes were then pulsed for 4 hr with 1 μCi [¹⁴C]leucine (protein) or 2 μCi [³H]thymidine (DNA), and the TCA precipitable counts analyzed using a Model LS-233 scintillation counter as described previously (13). Control cultures consisted of heat-killed (56°C, 30 min) lymphocytes or lymphocytes to which no drugs were added.

RESULTS

1. Inhibition of LT Secretion in Vitro by Primary Lectin-Stimulated Human Lymphocytes Treated with Colchicine, Cytochalasin B, Emetine, or Cycloheximide

Experiments were initiated to assess the effects of colchicine or cytochalasin B on the release of LT activity from lectin-stimulated human lymphocytes in vitro. Human tonsil or adenoid lymphocytes were activated with 20 μg/ml PHA-P in the presence of various concentrations of colchicine (a known inhibitor of microtubule formation) or cytochalasin B (a known inhibitor of microfilament formation) for 2–3 days at 37°C (17, 18). The supernatants were collected, dialyzed, and tested for toxic activity on L-929 cells, as described under Materials and Methods. Control supernatants were collected from cells cultured for 3 days without PHA or without PHA but with colchicine or cytochalasin B. These control supernatants (after dialysis) had essentially no detectable toxic activity. As can be seen in Fig. 1A, colchicine effectively inhibited LT release from primary PHA-activated human lymphocytes in vitro. Nearly 100% reduction of control LT activity was obtained at 10⁻³ M colchicine, while 80% reduction occurred at 10⁻⁴ M. However, this reduction in the release of supernatant LT activity was not due to cell death, since both control and colchicine-treated cells showed equally high viabilities and total cell counts. Similar results, shown in Fig. 1B, were also obtained using another inhibitor of secretion, cytochalasin B. This drug effectively blocked LT release at concentrations as low as 1 μg/ml, supporting the previous findings of Kramer and Granger (18). Emetine, an irreversible inhibitor (22), and cycloheximide, a reversible inhibitor, were also tested for their effects on the release of LT activity from primary activated lymphocytes in vitro. Table 1 demonstrates that emetine and cycloheximide totally suppressed release of LT activity after 14 hr of stimulation at levels which fully inhibited lymphocyte protein synthesis, as shown in Figs. 1C and D. Furthermore, inhibition of LT release by emetine appeared to be irreversible, since lymphocytes treated with 10⁻⁴ M emetine for 3 hr, washed, and restimulated with PHA could not release LT activity into the supernatants (Table 1). In contrast, lymphocytes treated with cycloheximide could release LT activity after washing. This is consistent with the fact that cycloheximide is a reversible inhibitor of protein synthesis.

Previous studies have shown that mitogen-activated human lymphocytes can possess high levels of intracellular toxic activity, while the intracellular levels in unstimulated cells are virtually undetectable. We have shown that this material resembles human LT, both physically and immunologically (2). In five separate and identical experiments, when fresh, unstimulated human lymphocytes were activated with lectins in the presence of 10⁻³ M colchicine, intracellular levels of toxic activity (LT) accumulated to 70–88% of the intracellular levels in untreated control cells, but release of LT into the supernatant was drastically reduced (Fig. 2). Thus, in the presence of colchicine, PHA-activated lymphocytes can apparently
synthesize and accumulate intracellular LT but are unable to release it into the supernatant.

II. The Effect of Colchicine and Emetine on LT Release by Lectin-Activated Prestimulated Human Lymphoid Cells in Vitro

Human adenoid lymphocyte suspensions were incubated in the presence of 10 μg/ml Con A for 3 days. After this period, the cells were washed and resuspended in MEMS with or without colchicine or emetine. The inhibitors were removed after 3 hr by additional washing, and the cells resuspended in MEMS plus 10 μg/ml Con A. Supernatants were collected, dialyzed to remove the inhibitors, and assayed for units of LT activity/ml, as described under Materials and Methods. The results of one of three experiments with essentially identical results are shown in Table 2. It is clear that these agents did not affect LT release from preactivated human lymphocytes.


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TABLE I

Effects of Emetine or Cyclohexamide on the Release of LT Activity by Primary Lectin-Stimulated Human Lymphocytes in Vitro

<table>
<thead>
<tr>
<th>Treatment of lymphocytes</th>
<th>Lectin</th>
<th>Percentage viability</th>
<th>LT activity (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>+</td>
<td>80</td>
<td>28 ± 4</td>
</tr>
<tr>
<td>Untreated</td>
<td>-</td>
<td>78</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Emetine (10⁻⁶ M)</td>
<td>+</td>
<td>74</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>Emetine (10⁻⁴ M)</td>
<td>+</td>
<td>66</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Emetine (10⁻⁴ M) washed</td>
<td>+</td>
<td>70</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Cyclohexamide (5 µg/ml)</td>
<td>+</td>
<td>69</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Cyclohexamide (5 µg/ml) washed</td>
<td>+</td>
<td>74</td>
<td>20 ± 2</td>
</tr>
</tbody>
</table>

* Human adenoid lymphocytes were obtained and stimulated with 20 µg/ml PHA-P in the presence or absence of various concentrations of emetine or cyclohexamide for 14 hr at 37°C. To test the reversibility of these drugs, lymphocytes were treated for 3 hr with the drug, washed with MEMS (three times, using 50 ml each), then activated with PHA-P for 14 hr at 37°C. Supernatants were collected, dialyzed against 1000 vol of PBS at 4°C, and tested for LT activity, as described under Materials and Methods.

III. The Effect of Colchicine or Emetine on Lectin-Induced Human Lymphocyte-Mediated Cytotoxicity (LICC) of L-929 Cells Using Unstimulated Human Lymphocytes in Vitro

The capacity of colchicine or emetine to effect LICC in vitro was next investigated. Fresh, unstimulated human adenoid lymphocytes were treated with various concentrations of colchicine or emetine for 3 hr at 37°C, washed, and placed on L-929 cells at various Ly:TGC ratios in the presence of Con A for 20 hr at 37°C. Untreated control cells received Con A or Con A plus a competitive binding agent.

![Fig. 2. Effect of colchicine on the biosynthesis and release of LT activity by lectin activated human lymphocytes in vitro.](image)

Human adenoid lymphocytes were incubated with 10⁻³ M colchicine or MEMS (control) in the presence of 20 µg/ml PHA-P. After 3 days at 37°C, the supernatants were collected, dialyzed, and tested for LT activity as described under Materials and Methods. Intracellular LT was obtained after lymphocytes (10⁶) were washed three times, using 25 ml MEMS for each wash; cells were resuspended in 4 ml of fresh MEM and sonicated for 6 min. This procedure lysed >99% of the cells by microscopic observation. The cellular debris was then removed by centrifugation, the supernatant was dialyzed, and LT activity was tested as described under Materials and Methods. Supernatant LT activity is expressed in Units/ml, while intracellular LT activity is expressed as Units/10⁶ lymphocytes/ml.
TABLE 2

Release of Soluble LT Activity from Con A-Preactivated Human Lymphocytes Treated with Emetine or Colchicine in Vitro

<table>
<thead>
<tr>
<th></th>
<th>Percentage lymphocyte viability</th>
<th>LT activity (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A (control)</td>
<td>80</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>Colchicine (10^{-3} M) + Con A</td>
<td>67</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>Emetine (10^{-4} M) + Con A</td>
<td>66</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>Emetine (10^{-6} M) + Con A</td>
<td>78</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>Emetine (10^{-8} M) + Con A</td>
<td>80</td>
<td>10 ± 1</td>
</tr>
</tbody>
</table>

* Human adenoid lymphocytes were obtained and preactivated for 3 days with Con A (10 μg/ml). The cells were then collectively washed and resuspended in MEMS with or without the presence of inhibitors. After 3 hr at 37°C, the cells were washed (three times, using 50 ml MEMS each) and placed into fresh MEMS with 10 μg/ml Con A for an additional 14 hr. Supernatants were collected, dialyzed against 1000 vol of PBS, and assayed for LT activity as described under Materials and Methods.

inhibitor, MAM, for 20 hr at 37°C. As can be seen in Figs. 3A and B, colchicine, or emetine treatment of these lymphoid cells dramatically inhibits their capacity to induce LICC in this system. Inhibition of LICC by colchicine or emetine was a dose-related phenomenon with maximal inhibition occurring in 10^{-3} M colchicine or 10^{-4} M emetine. Additional experiments, shown in Figs. 3A and B, revealed that 100 μl of heterologous rabbit antisera directed at the 70,000–90,000 MW class of human α-lymphotoxins released by activated human lymphocytes in vitro (anti-α-LT) also blocked LICC in this system.

IV. Effect of Colchicine or Emetine on LICC Using Mitogen-Preactivated Human Lymphocytes in Vitro

The following experiments were conducted to test whether lectin-prestimulated lymphocytes could induce LICC, and whether this activity could be inhibited by colchicine or emetine. Additional experiments were also conducted to examine the effect of anti-α-LT sera on these reactions. Mitogen (PHA, Con A)-preactivated human lymphocytes (Day 3) were treated with 10^{-3} M colchicine or 10^{-4} M emetine for 3 hr, washed, and subsequently employed in direct LICC of L cells using soluble Con A as the inducing agent. As can be seen in Fig. 4, colchicine, causes only slight inhibition of LICC when preactivated human lymphocytes are employed as effector cells. In addition, 10^{-4} M emetine was ineffective at blocking LICC when used at levels which inhibit protein synthesis 98% (Fig. 1C). In contrast, LICC induced by either emetine- or colchicine-treated Con A-preactivated effector lymphocytes was inhibited by heterologous anti-α-LT antisera. These experiments have been performed three separate times employing effector lymphocytes obtained from tonsils or adenoids with identical results.

V. The Relationship of Intracellular LT and Protein Synthesis to the Capacity of Activated Human Lymphocytes to Release LT and to Cause LICC

It seemed plausible that the capacity of preactivated lymphocytes to cause target cell destruction in the presence of inhibitors of protein synthesis could be due to
FIG. 3. Effect of colchicine or emetine on lectin-induced cytotoxicity (LICC) of L-929 cells by primary unstimulated human lymphocytes in vitro. A. Human adenoid lymphocytes were obtained and treated with various concentrations of colchicine for 3 hr at 37°C. The cells were then washed with PBS, and various numbers placed on 10^6 L-929 target cells, in the presence of Con A or Con A plus a competitive binding inhibitor, MAM, as described under Materials and Methods. To one set of tubes, 100 μl of rabbit anti-α-LT serum was added, while 100 μl normal rabbit serum (NRS) was added to the controls. After 18 hr at 37°C, the viable adherent L. 929 cell number was determined on a Coulter Counter. ● Untreated control lymphocytes + Con A ± NRS. ○, Untreated control lymphocytes + Con A ± α-MAM. △, Lymphocytes treated with 10^-3 M colchicine + Con A. ▲, Lymphocytes treated with 10^-3 M colchicine + Con A. A, Untreated control lymphocytes + Con A + anti-α-LT serum. B. Lymphocyte suspensions were treated with various concentrations of emetine by procedures identical to those employed for colchicine, as described in A. ▲, Untreated control lymphocytes + Con A ± NRS. ●, Untreated control lymphocytes + Con A + anti-α-LT serum. ○, Lymphocytes treated with 10^-4 M emetine + Con A. △, Lymphocytes treated with 10^-4 M emetine + Con A.

accessibility to preformed pools of a cytotoxic effector(s), such as LT. To test this concept, Con A-activated lymphocytes (Day 3) were collected, washed, and placed into fresh medium containing 2 × 10^-5 M emetine plus 5 μg/ml Con A to promote release in the absence of cellular protein synthesis, or emetine without Con A for 12–15 hr at 37°C. These cells were then collected and washed, and their cytolytic potential was tested on target L cells in the presence of fresh Con A. The results of one of two experiments with similar results is shown in Table 2. Table 2A indicates that preactivated lymphocytes cultured with emetine and Con A, for 18 hr, had a much reduced capacity to cause target L-cell lysis than cells cultured in emetine alone. When intracellular levels of LT activity were analyzed, it was found
that lymphocytes cultured in the presence of Con A alone had high levels of intracellular toxic activity. In contrast, in the presence of emetine, Con A activation dramatically reduced these intracellular levels (Table 3). It should be noted that both emetine-treated and nontreated cells released similar levels of LT activity when preformed LT pools were intact; however, further studies, not reported here, revealed that once the pools in emetine-treated cells were exhausted, release stopped. These experiments suggested that inhibition of protein synthesis blocked replenishment of intracellular or membrane-associated pools, but did not block release of these pools into the supernatant. There was also a correlation that once intracellular pools are exhausted, these cells can no longer release LT or function as effectors of LICC.

VI. The Relationship among Membrane-Associated LT Forms, Active Membrane Secretion, and LICC Mediated by Lectin-Preactivated Human Lymphocytes in Vitro

A recent report revealed that LT molecules can be expressed on the plasma membranes of lectin-activated human lymphocytes in vitro (17). We decided to test whether these molecules could be released from the membrane in the absence of active secretion and how this related to LICC. Three-day Con A-preactivated human lymphocytes were collected, washed, and treated with $10^{-3} M$ colchicine for 3 hr at 37°C, washed and then stimulated with Con A for 12 hr. After this time, the cells were again washed and restimulated with fresh Con A for a final 12 hr. At each time point, supernatant LT activity, LICC, and intracellular LT were assayed on L-929 cells in vitro. Shown in Table 4 are the results of two experiments yielding very similar results. When 3-day Con A-activated human lymphocytes are treated with $10^{-3} M$ colchicine as above, then stimulated with Con A for 12 hr, their

![Graph](image-url)
TABLE 3
Effect of Prolonged Incubation with Con A on the Lytic Activity and Intracellular LT Activity of Emetine-Treated Human Effector Lymphocytes in Vitro* A. MICC

<table>
<thead>
<tr>
<th>Percentage L-cell viability</th>
<th>Control untreated</th>
<th>Emetine treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ly:TGC</td>
<td>+ Con A</td>
<td>− Con A</td>
</tr>
<tr>
<td>0.2:1</td>
<td>19 ± 3</td>
<td>27 ± 4</td>
</tr>
<tr>
<td>2:1</td>
<td>4 ± 1</td>
<td>9 ± 2</td>
</tr>
</tbody>
</table>

| B. LT activity | | | |
| Treatment | Percentage viability | Intracellular LT (units/10^6 cells/ml) | Percentage reduction |
| Con A | 70 | 166 ± 10 | — |
| Emetine (5 × 10^{-6} M) + Con A | 60 | 15 ± 3 | 91 |
| Emetine (5 × 10^{-6} M) − Con A | 65 | 110 ± 7 | 33 |

* Con A-stimulated (Day 3) human adenoid lymphocytes were obtained, washed with PBS, and resuspended in fresh MEMS with or without 5 μg/ml Con A and with or without 5 × 10^{-6} M emetine. After 18 hr at 37°C, the lymphocytes were washed with PBS, and various numbers placed on 10^6 L-929 TGC in the presence or absence of 5 μg/ml fresh Con A (MICC). To test for intracellular LT activity, 10^5 lymphocytes were washed extensively with PBS and sonicated in 3 ml of MEMS. The cellular debris was then removed by centrifugation and LT activity tested as described under Materials and Methods. Error values represent standard deviation of duplicate determinations.

The cellular processes involved both in LT release and in causing LICC of L-929 cells in vitro appear to be modulated by the state of effector lymphocyte activation. Levels of both emetine and cycloheximide, which were shown to inhibit lymphocyte protein synthesis, totally blocked LT biosynthesis, accumulation into intracellular pools, and release into the supernatant by primary lectin-treated human lymphoid cells. The capacity of primary lymphocytes to induce LICC of L cells was also completely blocked by these same inhibitors. Inhibitory effects on LICC and LT release were not due to killing of the effector cell. Microscopic observation revealed that this treatment did not interfere with contact between aggressor lymphocyte and target cell in the LICC reactions. It should be noted that increased levels of emetine and cycloheximide were required to suppress LICC of L-929 cells mediated by human lymphocytes, as compared to the levels reported to inhibit immune murine T-lymphocyte cell killing in vitro (23). Cytochalasin B and
colchicine, agents which have been shown to disaggregate intracellular microfilaments and microtubules and block the movement of intracellular vesicles to the plasma membrane, also inhibited LT release in these cultures. These studies revealed colchicine did not effect LT biosynthesis itself, but only its release, for we could detect significant intracellular but not supernatant LT activity in drug-treated lectin-stimulated cells. In addition, colchicine-treated cells were unable to induce LICC of L-929 cells in vitro. In contrast, 3-day lectin-prestimulated cells, when treated with either emetine or colchicine, were as effective as untreated controls in either releasing LT upon restimulation with PHA or causing LICC of L-929 cells. Controls confirmed that the observed effects were not due to loss of lymphocyte viability caused by any of the inhibitor treatments.

It appears that preactivated human lymphoid cells, in the presence of lectin, can release LT from intracellular pools without further protein synthesis and can release membrane-associated LT forms in the absence of vesicle secretory mechanism(s). Previous studies revealed that human lymphocytes stimulated with lectins for 2 or 3 days in culture develop high intracellular levels of LT (2), and that these cells, including T cells, express α-LT immunologic determinants on their plasma membranes (17). Additional studies also indicated that 2- to 4-day lectin-preactivated cells are capable of very rapid LT release upon lectin restimulation (16). The present studies reveal that 2- to 3-day lectin-preactivated cells can release LT upon restimulation in the absence of cellular protein synthesis. We have observed similar results employing purified human T cells (P. Harris, T. Benvenuti, and G. Granger, unpublished results). It is possible this material is released by inhibitor-treated cells from preformed intracellular pools. In support of this suggestion, repeated lectin stimulation of emetine-treated cells revealed that as LT appeared in the supernatant, the LT in intracellular pools was depleted, and the capacity of the cells to release material also declined. However, it is surprisingly difficult to deplete the intracellular LT pools with lectin stimulation. It is clear that

| TABLE 4 |
|-------------------------------|-------------------|-------------------|-------------------|
| **Inability of Lectin-Preactivated Human Lymphocytes to Cause MICC or Release LT Activity after Treatment with Colchicine plus Con A** |
| First 12-hr incubation with Con A | Second 12-hr incubation with Con A |
| Percentage lymphocyte viability | LT activity (units/ml) | MICC (%) | Percentage lymphocyte viability | LT activity (units/ml) | MICC (%) | Intracellular LT activity |
| Control | | | | | | |
| Colchicine (10⁻³ M) | 74 | 21 ± 3 | 82 | 66 | 4 ± 2 | 12 | 60 ± 6 |

* Human adenoid lymphocytes were preactivated for 3 days at 37°C, with 10 μg/ml Con A. These cells were collected, washed, and treated with 10⁻³ M colchicine for 3 hr at 37°C, then washed and restimulated with 10 μg/ml Con A for 12 hr (first 12-hr incubation with Con A). The cells were then collected, washed, and again stimulated with 10 μg/ml Con A for a final 12-hr period (second 12-hr incubation with Con A). During each of these incubations, the lymphoid cells were tested for their ability to cause MICC or to release LT activity, as described under Materials and Methods.

MICC was determined by the percentage lysis of L-929 cells at a 10:1 ratio during an 18-hr incubation at 37°C.

Intracellular LT activity is expressed as Units LT activity/10⁶ lymphocytes/ml.
lectin-pretreated cells could release LT in the apparent absence of vesicle-associated secretory processes. The LT released into the supernatant under these conditions did not appear to originate from intracellular pools, because upon examination, we found they were not depleted upon multiple lectin restimulation. These results imply two sources for LT released under these conditions: (a) material released directly from the plasma membrane; and (b) secretion of intracellular material, by a non-microtubule- or microfilament-dependent process. We presently favor the former concept, namely, shedding from the plasma membrane, since intracellular pools remained high. However, we cannot discount the possibility that pools were replenished by intracellular protein synthesis. Additional experiments will be required to clarify this situation.

The present results allow formulation of a model conceptualizing lymphocyte activation, LT biosynthesis, expression on the cell surface, and release into the supernatant. The activation of a primary cell with lectins induces LT biosynthesis and accumulation into intracellular pools. This initial biosynthetic step can be blocked by inhibitors of protein synthesis. Once intracellular pools have formed, LT molecules can be expressed on the activated lymphocyte plasma membrane. A vesicle-associated secretory system appears to be required to release LT from intracellular pools. However, once LT molecules are expressed on the cell surface, they can be shed or released directly into the supernatant by lectin stimulation without further cellular protein synthesis or without cellular secretory processes. This latter situation is further supported by our previous findings that once LT molecules have been expressed on the activated cell surface (at 2–3 days), intracellular levels rapidly decline; however, LT release, upon restimulation of these cells with lectins, can be very rapid (2, 16). This model would explain why the effect of a particular inhibitor on release of soluble LT molecules in this culture system depends upon the particular stage of activation of the lymphocyte. In addition, these data support the concept that once activated, the cell is armed with LT molecules that are available for release or delivery via two separate pathways: (a) classical secretion from intracellular pools; and (b) a shedding or release directly from the membrane of the activated cell. It should be emphasized that both these release pathways have been shown to operate only while the activating agent is in contact with the lymphoid cell membrane receptor(s) (16).

There is substantial evidence to support the concept that LT molecules are lytic effectors of LICC-induced lysis of murine L-929 cells mediated by human lymphocytes in vitro. It is clear that L-929 cells selected in vitro for sensitivity to human α-LT are also highly sensitive to direct human lymphocyte LICC (24). Kramer and Granger found the LICC effector human lymphocyte deposits material(s) on the target L-929 cell surface by direct cell contact during the lymphocyte-dependent phase of this reaction, which can cause cell lysis during the lymphocyte-independent phase, for target L-cell lysis can be blocked after interaction with the aggressor lymphocyte by trypsin treatment or by lowering the temperature from 37 to 34°C (25). Human α-LT bound to the L-cell surface can be inhibited in vitro by the same treatment (25). Hiserodt and Granger reported that anti-human LT sera will block human T-lymphocyte-mediated LICC L-929 cell lysis when added at the actual lytic or lymphocyte-independent phase, which eliminates the possibility of antiserum blocking of cytolysis by interfering with recognition or cellular contact (20). Moreover, these antibody blocking studies supported the concept of two forms of delivery of LT molecules to the L-929 cell by
the lectin-activated human lymphocyte: (a) direct delivery by membrane contact; and/or (b) release of LT into the supernatant. The present studies reveal that there is a direct correlation between the capacity of a lymphocyte to induce LICC of L-929 cells and its capacity to synthesize and release or shed LT. Clearly, the present studies suggest an interesting situation, for once activated, a lymphocyte may deliver LT to a target cell by a secretory process in the absence of protein synthesis, or perhaps by a direct transfer of a membrane-associated form in the complete absence of biosynthesis or secretion.

The study of CMI cell-lytic reactions and the LT system of cell-lytic molecules are both complex problems, and the role of this system in CMI reactions is not clear. CMI reactions involve multiple types of effector cells, and therefore there exists the possibility of distinct types of cell-lytic mechanism(s). Biochemical evidence clearly reveals that LT molecules from both humans and experimental animals form a complex but interrelated system of cell toxins. Functional studies of human LT molecules indicate the smaller, most commonly studied LT forms are only weakly cell lytic or growth inhibitory to most cells in culture, except for the highly LT-sensitive L-929 cell (10). In contrast, the high MW forms can cause rapid $^{51}$Cr release (4–9 hr) from many cell types, and both human and murine Cx forms released by alloimmune cells can cause selective destruction of specific target cells in vitro (10). When we view the above results collectively, we feel that LICC of L-929 targets is a unique in vitro cell killing system. Because the L-929 cell is uniquely sensitive to the smaller LT forms, lysis can be mediated in this LICC system by these forms which may not be operative on another more LT-resistant cell type. Our own bias is that if involved in CMI lytic reactions, the highly effective complex forms are probably the more likely candidates as lytic effectors. This is indeed supported by extensive experiments which reveal that antisera reactive with the Cx forms are potent inhibitors of various CMI reactions, whereas antisera reactive with smaller forms are at best only weakly effective (26). Previous results revealed that protein synthesis was necessary for LT release by primary lectin-stimulated human lymphocytes in vitro (13). This finding has been pointed out as incongruous with the concept that these molecules may be employed as lytic effectors by murine T lymphocytes, because the latter cells cause target cell destruction in the absence of protein synthesis (14, 15). The present results clearly indicate that activated human lymphoid cells can become “armed” by synthesis of LT molecules which can then be delivered by secretion or perhaps by release from the plasma membrane in the absence of protein synthesis. Indeed, part of the differentiation associated with the generation of functional CTL upon antigen stimulation may be to develop intracellular pools and membrane-associated forms of these molecules.

REFERENCES