MECHANISMS OF LYMPHOCYTE-MEDIATED CYTO-TOXICITY .2. BIOCHEMICAL AND SEROLOGIC IDENTIFICATION OF A PRECURSOR LYMPHOTOXIN FORM (PRE-LT) PRODUCED BY MLC-SENSITIZED HUMAN LYMPHOCYTES-T INVITRO

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JOURNAL OF IMMUNOLOGY, 126(5)

0022-1767

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1981

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Peer reviewed
MECHANISMS OF LYMPHOCYTE-MEDIATED CYTOTOXICITY

II. Biochemical and Serologic Identification of a Precursor Lymphotxin Form (pre-LT)
Produced by MLC-Sensitized Human T Lymphocytes In Vitro.1

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The biochemical and immunologic properties of a cell toxin(s) released into the fluid phase and expressed on the surface of lectin-activated alloimmune human cytotoxic lymphocytes has been investigated. The results indicate that the initial cytotoxin(s) released into the supernatant from alloimmune lymphocytes represents a precursor form of lymphotxin (pre-LT), and in this precursor form, the \( \alpha \) and \( \beta \) antigens exist as masked or cryptic determinants. The pre-LT form in unfraccionated supernatants was defined immunologically by neutralization with a polyspecific anti-LT antisera (anti-WS), and its lack of reactivity with anti-\( \alpha \) and anti-\( \beta \) LT antisera. However, gel filtration chromatographic profile of the pre-LT cytotoxic activity was heterogeneous and showed multiple m.w. classes that were characteristic of the chromatograms of traditionally defined LT. These fractionated components were now readily neutralized with anti-\( \alpha \) LT antiserum. In addition, the pre-LT cytotoxic activity in unfraccionated supernatants treated by various physical or chemical means rendered the cytotoxic activity neutralizable by an anti-\( \alpha \) LT antiserum, indicating an immunologic relationship between the pre-LT form and components of the LT system. The antigens associated with pre-LT form were detectable, in part, on the LT-Cx and \( \gamma \) LT molecules by a fluid phase immunoadsorption assay.

A functional immunoadsorption assay was used to detect the antigenic determinants of the pre-LT form on the cell surface of alloimmune lymphocytes before and after lectin activation. \( \alpha \) LT-associated antigens were detectable on cytotoxic effectors only after lectin activation. These results imply LT may function as cell surface delivered cytotoxic molecules. LT activity was not detected in supernatants obtained from alloimmune cytotoxic reactions, however, the pre-LT and \( \alpha \)-LT neutralizing activities of the anti-LT sera were significantly diminished after the incubation of these sera in the cytotoxic reaction and indicated that LT molecules were produced during the cytotoxic reaction, and LT remained closely associated with the lymphocyte:target cell conjugates.

The relationship of the pre-LT form to the capacity of the various anti-LT antisera to affect the human alloimmune cytotoxic reaction is discussed. The results provide additional support to the concept that the LT system is involved in the lytic mechanism of cytotoxic T cells.

In the preceding article, we investigated the effects of a variety of anti-human lymphotxin antisera (anti-LT) in a specific human alloimmune cytotoxic reaction system in vitro (1). The capability of an antiserum to inhibit this cytotoxic system depended on the antigenic specificity recognized by the particular anti-LT antiserum. Anti-LT sera of restricted specificity, i.e., reactive with the \( \alpha \) or \( \beta \) LT m.w. classes, were not inhibitory in this system. In contrast, the polyspecific anti-whole supernatant (anti-WS), an antiserum capable of neutralizing the lytic activity of all presently identified LT components, was a potent inhibitor of lysis.

A number of possibilities exist that may account for the inability of the anti-\( \alpha \) or anti-\( \beta \) antisera to inhibit the alloimmune cytotoxic reaction. One explanation would provide no role for LT molecules in the lytic mechanism employed by these effector cells (2). A second alternative is that the \( \alpha \) and \( \beta \) antigenic determinants are in some fashion inaccessible to the inhibitory anti-LT antibodies. A corollary of the second possibility is that antibodies reactive with an additional LT-associated antigenic determinant(s), distinct from the \( \alpha \) or \( \beta \) determinants, may be required to inhibit lymphocyte-mediated cytototoxicity.

The results presented in this manuscript will provide evidence in support of the latter possibility. The evidence was obtained from our studies on the biochemical and immunologic properties of LT released by alloimmune populations of human lymphoblasts in response to stimulation with mitogenic lectins. The results indicate that when LT activity is released into the fluid phase by the stimulated alloimmune effector cells, LT activity exists in a precursor form in which the \( \alpha \) and \( \beta \)-associated antigens are masked. Furthermore, the polyspecific anti-LT antiserum contains neutralizing antibodies against this precursor-LT form, and these antibodies may account for the inhibitory action of these antisera in the human alloimmune cytotoxic reaction.

MATERIALS AND METHODS

Medium and chemical reagent. Tissue culture medium for routine passage of target cells or lymphocytes was RPMI 1640 (Grand Island Biological Co. (GIBCO), Grand Island, NY), supplemented with 10%, (v/v) heat-inactivated fetal bovine serum (GIBCO) and antibiotics (1). Potassium chloride was reagent grade (Sigma, St. Louis, MO) and urea was ultrapure grade (Swaert-Mann, Orangeburg, NY). The nonionic detergent Nonidet P-40 (NP-40) (Particle Data Laboratories, Ltd., Elmhurst, IL) was dissolved in phosphate-buffered saline (PBS) (0.15 M NaCl, NaHPO4 0.01 M, pH 7.2). \( \alpha \)-Methyl-D-mannoside, a competitive binding inhibitor of concanavalin A (Con A) was obtained from Sigma.

LT assay and anti-LT antisera. LT activity was measured on 1 \( \times \) 10^6 mitomycin-C treated murine L-929 cells as previously described (3). The unit(s) of LT activity per milliliter in the original preparation was defined as the reciprocal dilution effecting a 50% reduction of the target L-929 cell...
mixed lymphocyte culture (MLC) and lectin activation of allolymphocyte effectors. Human peripheral blood or adenoid lymphocytes were primed in a one-way MLC with mitomycin-C treated WI-L2 lymphoblastoid cell (20:1 ratio) for 5 to 7 days as described (1). Lymphocytes harvested from the MLC cultures were washed twice with 50 vol of medium and resuspended at 1 x 10^6 cells/ml in medium containing Con A (Sigma) at 10 μg/ml. These cultures were incubated for 5 to 20 hr at 37°C.

Procedure is referred to as MLC-primed lectin-activation.

Isolation of human T Cells. Populations of Ig-negative human T-enriched lymphocytes were purified from freshly isolated peripheral blood lymphocytes (PBL) by rosetting formation with sheep red blood cells (SRBC), as previously described (6). This procedure yielded populations of T cells that were 90 to 95% pure, as judged by expression of Ig and rosetting with SRBC. Lymphocyte-bound SRBC were removed by distilled water lysis.

Measurement of membrane-associated LT activity. These experiments were performed as previously described (6). Briefly, MLC-primed lectin-activated PBL lymphocytes were washed twice with 50 vol of cold medium containing 10 mM α-methyl-D-mannoside. Anti-LT sera were then added to the lymphocytes with lectin. High levels of cytotoxic activity (pre-LT) released by MLC-primed lectin-activated lymphocytes was concentrated on a 2.5- x 55-cm column containing Ultrogel AcA 44 (LKB, Uppsala, Sweden). Elution was carried out in 10 mM Na phosphate, pH 7.0, 10 mM EDTA buffer at a flow rate of 20 ml/hr. One hundred- to 200-μl aliquots of every other fraction were tested for cytotoxic activity on L-929 cells. Where indicated, fractions corresponding to the various LT activity peaks were pooled, reconstituted to 150 mM NaCl, and concentrated by ultrafiltration (PM-10).

Measurement of membrane-associated LT. Recent studies have shown that the LT system is composed of at least two components, α and β, each being represented by multiple isotypes. The LT system is involved in the regulation of inflammation and immune responses, and its understanding is crucial for the development of targeted therapies. Therefore, the identification of an immunologic precursor form of human LT (pre-LT) released by MLC-primed lectin-activated lymphocytes has been investigated. Preliminary experiments revealed that after stimulation in MLC, human and murine lymphocytes showed an enhanced capacity to release cytotoxins in vitro in response to lectins, when compared to non-MLC stimulated controls. The release of cytotoxic activity by MLC-lymphoblasts was very rapid with detectable levels (5 to 10 hr) in culture. This finding indicates that the cytotoxic activity of the lymphocytes is not due to the presence of preformed cytotoxins in the cell. The area of the cytotoxic activity peaks revealed the LT-Cx. The area of the cytotoxic activity was tested for neutralization of the cytotoxic activity obtained from MLC-primed lectin-activated lymphocytes. Small amounts (less than 15 U) of the cytotoxic activity were tested for neutralization in these experiments to assure that the quantities of the cytotoxic activity released from the MLC-primed lectin-activated lymphocytes are presented in Table 1 (Fig. 2). The area of the cytotoxic activity peaks revealed the LT activity obtained from MLC-primed lectin-activated lymphocytes was tested for neutralization of the cytotoxic activity obtained from MLC-primed lectin-activated lymphocytes. Small amounts (less than 15 U) of the cytotoxic activity were tested for neutralization in these experiments to assure that the quantities of the cytotoxic activity released from the MLC-primed lectin-activated lymphocytes are presented in Table 1. The nonneutralizable portion of the cytotoxic activity in the β LT m.w. class most likely represents the non-α LT cross-reacting β-LT subclass (4, 7). The instability of the cytotoxic activity of the γ LT kienas prohibited testing of this fraction. Control experiments in which whole supernatant cytotoxic activity was dialyzed against the column buffer (0.01 M phosphate, 10 mM EDTA, 0.15 M NaCl) showed no change in the antigenic properties of these cytotoxic(s) and no decrease in the level of the cytotoxic activity was observed when compared to untreated freshly isolated whole supernatants. Passage of the unfractionated whole supernatant over goat anti-α LT I gG covalently linked to Sepharose 4B did not retain the cytotoxic activity found in these supernatants (data not shown).

These data indicated the cytotoxic activity released by MLC-primed lectin-activated PBL was biochemically and antigenically related to the known components of the LT system. This suggested results were obtained with the goat anti-WS or anti-α LT sera when tested for neutralization of the cytotoxic activity obtained from MLC-primed lectin-activated lymphocytes. Small amounts (less than 15 U) of the cytotoxic activity were tested for neutralization in these experiments to assure that the quantities of the cytotoxic activity released from the MLC-primed lectin-activated lymphocytes are presented in Table 1. The nonneutralizable portion of the cytotoxic activity in the β LT m.w. class most likely represents the non-α LT cross-reacting β-LT subclass (4, 7). The instability of the cytotoxic activity of the γ LT kienas prohibited testing of this fraction. Control experiments in which whole supernatant cytotoxic activity was dialyzed against the column buffer (0.01 M phosphate, 10 mM EDTA, 0.15 M NaCl) showed no change in the antigenic properties of these cytotoxic(s) and no decrease in the level of the cytotoxic activity was observed when compared to untreated freshly isolated whole supernatants. Passage of the unfractionated whole supernatant over goat anti-α LT IgG covalently linked to Sepharose 4B did not retain the cytotoxic activity found in these supernatants (data not shown).
The capacity of rabbit anti-LT to neutralize the cytotoxic activity of pre-LT after fractionation by gel filtration chromatography*

<table>
<thead>
<tr>
<th>Antiserum Tested</th>
<th>LT-Cx region (Fx No. 32)</th>
<th>% Neutralization</th>
<th>LT region (Fx No. 34)</th>
<th>% Neutralization</th>
<th>LT region (Fx No. 44)</th>
<th>% Neutralization</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRS</td>
<td>2</td>
<td>0</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-WS</td>
<td>95 ± 7</td>
<td>97 ± 5</td>
<td>102 ± 7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-α-LT</td>
<td>80 ± 2</td>
<td>100 ± 10</td>
<td>47 ± 12</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Peak fractions taken from the gel filtration chromatogram shown in Figure 2 were employed as a source of the indicated LT m.w. class. Two hundred microfilters (representing for the LT-Cx class 2 units of activity; α and β-LT regions, 5 units each) of the indicated fraction was incubated with 200 μl of NRS, anti-WS, or anti-α-LT, and then 100 μl were tested in duplicate for residual cytotoxic activity on L-929 target cells. The percentage of neutralization was determined relative to the activity of the untreated fraction. Data represent the mean ± range of duplicate samples.

α LT antigen form. However, the pre-LT antigenic form is stable to storage at least 3 to 4 days at 4°C. Treatment with high levels of detergent (0.5%) or urea (8 M) partially reduced the total level of cytotoxic activity in these preparations, and this effect is reflected in the calculations showing an apparent neutralization of LT activity by normal control serum.

Gel filtration chromatography of the detergent-treated supernatant revealed the LT-Cx, α, and β classes were present (data not shown). This result indicated that detergent treatment alone did not result in a selective loss of any LT m.w. class.

The association of pre-LT antigenic determinant(s) with the LT-Cx and γ LT m.w. fractions. In order to physically characterize the α-LT antigens associated with the pre-LT form, a fluid-phase immunoabsorption assay was employed to inhibit the pre-LT neutralizing antibodies present in the anti-WS serum. Chromatographically separated LT-Cx, α, β, and γ LT containing m.w. fractions obtained from MLC-primed lectin-activated PBL supernatants were tested for their capacity to abrogate the pre-LT neutralizing activity of anti-WS. To accomplish this, 200 μl of the various LT classes or unfractionated whole supernatant containing similar levels of cytotoxic activity (10 to 15 U) (LT-Cx and γ classes were pooled separately and concentrated by ultrafiltration), were mixed with 200 μl of a 1:3 dilution of rabbit anti-WS and incubated for 1 hr at 0°C. Various amounts of these mixtures were then tested for their capacity to neutralize an additional 5 U of pre-LT activity present in unfractionated supernatants obtained from the same MLC-primed lectin-activated PBL. The results of this experiment are presented in Figure 3. The preincubated LT-anti-WS mixtures were not cytotoxic for L-929 cells in the absence of additional LT. Antigens present in unfractionated whole supernatant completely abrogated the pre-LT neutralizing activity of anti-WS. In contrast, antigens associated with the α or β LT fractions showed no capacity to absorb pre-LT antibodies. However, LT-Cx and γ LT fractions contained antigens that partially abrogated the pre-LT neutralizing activity of the anti-WS serum. This suggested that LT-Cx or γ LT-like molecules may contain, in part, antigenic determinants associated with the pre-LT form.
lymphblasticoid cell line for 5 days. Lymphocytes from this effector population were activated with Con A for 10 hr or, as controls, cultured without Con A. These cells were then washed twice with cold medium containing 10 mM α-methyl-mannoside and subsequently used to absorb rabbit anti-WS or anti-αLT antiserum. The absorbed or, as control, unabsorbed antisera were then tested for their capacity to neutralize pre-LT in unfraccionated supernatant or an α LT preparation purified by gel filtration chromatography as described in Materials and Methods. The results of these experiments are presented in Figure 4. Both the anti-WS and anti-αLT antisera were significantly diminished in their anti-LT neutralizing activities when absorbed on Con A-activated alloimmune lymphocytes. The anti-αLT LT-neutralizing antibodies were not absorbed on non-lectin activated alloimmune lymphocytes. In contrast, the pre-LT neutralizing antibodies in the anti-WS were absorbed by non-activated alloimmune cells. This result indicated that an antigenic component(s) associated with LT activity was present on the surface membranes of alloimmune lymphocytes. Furthermore, this result suggested the existence of the pre-LT antigenic determinants was not due to possible artifacts resulting from Con A stimulation of these cells.

The capacity of anti-αLT LT antiserum and complement (C) to inhibit direct lymphocyte-mediated cytotoxicity of allogeneic WI-L2 target cells. The previous results indicated that a LT-associated antigen were detectable on the cell surface of these MLC-primed lectin-activated lymphocyte populations. The expression of a LT-associated determinant(s) may render the expressing cell susceptible to lysis by antibody and C. Therefore, if the cytotoxic effector cell in this system expressed an α LT determinant(s), the cells’ lytic activity should be diminished by treatment with anti-αLT LT plus C. Lectin-activated or nonlectin-activated alloimmune lymphocytes were treated with rabbit or goat anti-αLT LT sera and C for 1 hr at 37°C. As controls, identical cultures of lymphocytes were treated with normal sera and C or left untreated. After treatment and washing, these cells were then tested for their capacity to lyse 51Cr-labeled WI-L2 target cells at various lymphocyte:target ratios in a 3-hr assay. To prevent lectin-dependent cytotoxicity resulting from residual Con A, α-methyl-mannoside was included in the reaction mixture at 5 mM. The results of 2 such experiments are presented in Table III. Treatment with either rabbit or goat anti-αLT LT serum and C reduced the capacity of lectin-activated immune lymphocytes to lyse the sensitizing target cell, WI-L2 from 20 to 30%. Nonlectin-activated lymphocytes were not affected by treatment with anti-αLT LT and C. However, rabbit anti-αLT LT partially inhibited activated lymphocytes without the addition of C. These results indicated that an LT-like determinant(s) was expressed on the activated alloimmune killer cell surface.

Detection of pre-LT and αLT-associated antigens produced during the alloimmune cytotoxic reaction. Goat anti-WS or rabbit anti-αLT LT serum were incubated with either MLC-generated effector lymphocytes or effector lymphocytes plus sensitizing WI-L2 target cells under conditions similar to those employed in the 51Cr-release assay. A parallel 51Cr-release assay, (25:1 ratio, 6-hr assay), target cell lysis was inhibited 85% by anti-WS, although lysis of WI-L2 in the presence of anti-αLT LT was nearly 100%. After incubation in the cytotoxic reaction, the supernatant fluids from the cytotoxic assay containing anti-LT antisera were cleared of cells by centrifugation and tested for their capacity to neutralize pre-LT activity present in unfraccionated whole supernatants or chromatographically separated α LT. The results are presented in Figure 5. The data indicate that the LT-neutralizing capacity of both anti-LT sera for their respective antigens was greatly diminished when incubated in the presence of lymphocytes and target cells. A small decrease in LT-neutralizing activity was observed after incubation with lymphocytes alone when compared to untreated antisera. No loss in LT neutralizing was observed after incubation with target cell alone (data not shown). Other experiments were conducted to see if LT activity was detectable in the supernatant fluid (in the absence of antisera) after the cytotoxic reaction was completed. It was observed that essentially no soluble LT activity (<2 U) was detectable under these conditions. Although, an equivalent of approximately 10 U of pre-LT activity was absorbed by the antisera. Collectively, these data indicate that the LT activity is lost during the cytotoxic reactions were either closely associated with lymphocytes and target cells, or the biologic (cytotoxic) activity of LT-associated antigens were rapidly lost or inactivated when released into the fluid phase.

DISCUSSION

The present results have described a new form of human LT activity detectable in whole supernatants from lectin-activated alloimmune lymphoid cells. This LT form appears to represent a precursor of the classically defined components of the LT system (α, β, γ, etc.) as described by their elution from molecular sieving columns (8–10) and has been tentatively termed “pre-LT.”
TABLE III
Decrease in the cytolytic activity of activated alloimmune lymphocytes by treatment with anti-α2 LT and C*

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Ly:TCG Ratio</th>
<th>% Lysis of Wt-L2 after Treatment of Effector with</th>
<th>Anti-α2 LT</th>
<th>Anti-α2 LT + C</th>
<th>Anti-α2 LT</th>
<th>Anti-α2 LT + C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:2</td>
<td>12:1</td>
<td>37.0 ± 0.2</td>
<td>Anti-α2 LT</td>
<td>32.5 ± 6.0</td>
<td>Anti-α2 LT</td>
<td>31.3 ± 0.5</td>
</tr>
<tr>
<td>1:2</td>
<td>6:1</td>
<td>27.4 ± 2.2</td>
<td>Anti-α2 LT</td>
<td>31.3 ± 1.6</td>
<td>Anti-α2 LT</td>
<td>24.5 ± 3.0</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:1</td>
<td></td>
<td>19.2 ± 2.0</td>
<td>Anti-α2 LT</td>
<td>23.0 ± 1.0</td>
<td>Anti-α2 LT</td>
<td>21.6 ± 3.0</td>
</tr>
<tr>
<td>10:1</td>
<td></td>
<td>12.7 ± 0.6</td>
<td>Anti-α2 LT</td>
<td>14.9 ± 1.2</td>
<td>Anti-α2 LT</td>
<td>11.6 ± 0.1</td>
</tr>
</tbody>
</table>

* Human PBL were sensitized to Wt-L2 in MLT for 5 days. Effector cells were then cultured for 15 hr with or without Con A (10 μg/ml). These lymphocytes (2 × 10⁶ cells) were washed twice with medium containing α-methyl-mannoside (10 mM) and resuspended in a 1:2 dilution of either goat or rabbit anti-α2 LT serum. Guinea pig serum (1:10 dilution) was added where indicated as a source of C. Lymphocytes were allowed to incubate for 1 hr at 37°C and then washed twice with medium. These cells were then added to 10⁶ ³¹¹Cr-labeled Wt-L2 target cells to give the indicated lymphocyte/target cell ratio and incubated for 3 hr at 37°C. The cytotoxic reaction mixture contained 5 mM α-methyl-D-mannoside. Data represent the mean ± SD of triplicate samples.

Figure 5. Loss of LT neutralizing activity of anti-WS or anti-α2 LT after incubation in the human alloimmune cytotoxic reaction. A, MLC-generated effector cells (4 × 10⁶ cells) were incubated alone or with Wt-L2 target cells (25:1 ratio) in the presence of a 1:4 dilution of goat anti-WS for 6 hr at 37°C in a total volume of 0.6 ml. Lymphocytes and target cells were removed by centrifugation, and the supernatant fluid was then assayed for its capacity to neutralize 20 units of pre-LT activity in unfraccionated whole supernatant. Anti-WS after treatment with lymphocytes alone ( ), lymphocytes + target cells ( ), untreated, diluted 1:4 ( ), data represent the mean ± range of duplicate samples. B, same protocol as above, except lymphocytes and target cells were incubated with rabbit anti-α2 LT serum (1:3 dilution). The capacity to neutralize LT activity by this antisemur was measured with 2 units of α-LT that was partially purified by gel filtration chromatography as described in Materials and Methods. Anti-α2 LT after treatment with: lymphocytes alone ( ); lymphocytes + target cells ( ); untreated, diluted 1:3 ( ).

Initial findings leading to the detection of a precursor-LT form was suggested by experiments in which both the anti-α and β LT class and subclass antisera failed to neutralize the cytotoxic activity in unseparated supernatants released by MLC-sensitized lymphoblasts after stimulation with Con A (Fig. 1). However, a polyclonal anti-LT serum (anti-WS) completely neutralized the cytotoxic activity in supernatants obtained from these cells. These findings help to explain partially previous results that indicated supernatants from 3- to 5-day lectin-activated nonimmune lymphocytes contained LT activities that were immunologically heterogeneous (4, 5). The stability at 4°C of the pre-LT activity in the supernatants further distinguished this lytic activity from the cytotoxic activity of the unstable β LT component (7).

A relationship between the pre-LT cytotoxic activity in supernatants from alloimmune lymphocytes and the classical m.w. components of the LT system was established by biochemical and immunologic methods. The evidence suggested the pre-LT cytotoxic activity existed in the supernatant as a precursor form of other LT classes, rather than as a distinct "non-LT" cytotoxin. This conclusion was based on the following results. i) Gel filtration chromatography of pre-LT containing alloimmune supernatants revealed multiple m.w. components of cytotoxic activity as measured on the LT-sensitive L-929 cell (Fig. 2). The chromatographic profile of these supernatants is identical to chromatograms of LT activity obtained from nonimmune lectin-activated human lymphocytes, and it is these chromatograms that define m.w. classes of the LT system, i.e., LT-Cx, α, β, and γ (8). Moreover, additional studies revealed that the majority of supernatant cell-lytic activity was recovered from the sieving columns, indicating that this fractionation step was not preselecting a particular LT form. ii) Rabbit anti-α LT antisera was fully able to neutralize the cytotoxic activity in the various m.w. LT classes obtained from alloimmune supernatants after gel filtration (Table I). iii) Immunoblotting of the anti-WS with various m.w. LT classes obtained by gel filtration of pre-LT activity revealed the antigens associated with the pre-LT form resided in the LT-Cx and γ LT m.w. classes (Fig. 3). iv) Various chemical or physical treatments (detergent, urea, freeze-thawings) of the unfractionated supernatant containing the pre-LT form was sufficient to render the cytotoxic activity susceptible to neutralization by anti-α LT antisera (Table III). These findings established a relationship between the pre-LT supernatant form produced by alloimmune lymphocytes and the gel filtration m.w. classes of the classical LT system. Although the molecular relationships are not yet clear, the pre-LT supernatant form may represent a complex of the smaller m.w. classes that dissociated in the sieving columns or after treatment with dissociating agents (see Reference 14). However, definite conclusions as to the nature of the pre-LT form will have to await isolation of this particular form. Furthermore, these results suggest the antigens associated with the α and β LT m.w. classes probably exist in a masked or cryptic form when LT is initially released from lectin activated alloimmune lymphocytes.

Previous studies indicate materials with LT activity form a system of cell-lytic molecules (9). The smaller α, β, and γ forms can associate with other each other to form complexes, and the complexes may further associate with nonclassical antigen-binding receptors (10, 11). The relationship of pre-LT, as it exists in the unseparated supernatant, to the known LT components is not clear from the present results. It should be pointed out that the system described in this study to produce pre-LT activity from alloimmune cells is
similar to that employed by Hiserodt and Granger (11, 12) to produce specific antigen-binding forms of human and murine (LT). The relationship between the pre-LT form and the receptor-associated LT components has been revealed in another series of experiments reported elsewhere, which demonstrated the capacity of an anti-human F(ab')2, antisera to inhibit pre-LT activity, as well as the LT activity associated with the specific antigen receptor forms (14). In addition, those studies revealed receptor-LT forms are associated with the LT-Cx class and another form, termed α, of 150,000, m.w. These classes also express pre-LT associated antigenic determinants (14). The concept of a precursor form of LT implies that the pre-LT may exist as a molecular complex composed of an antigen receptor and lytic subunits, similar to the LT-Cx, and or the α, except that the α and β antigens are masked (9, 11). We do not think that the pre-LT represents a membrane fragment, since the activity associated with this pre-LT form is not pelleted at 100,000 x g for 1 hr. Further experiments are in progress to clarify this point.

We would suggest that the T cell is the major cell type producing the pre-LT form in these cultures. Other investigators have described LT production by isolated human T cell populations stimulated with lectins in vitro (15-17). Although unseparated populations of human lymphocytes were employed in the present study, we feel that the rapid and high levels of intracellular LT pools and rapidly release this material upon restimulation (18, 19), whereas, nonrestimulated cells lack these pools and release much more slowly in vitro. Thus, allolantinogenic stimulation in MLC has selectively increased both the effector cell numbers and the potential of the T cell clones in this population to rapidly release LT upon lectin stimulation. This concept is further supported by the finding of essentially identical LT forms derived from purified populations of human T cells (14).

The present results reveal the presence of lymphotixin-associated antigen determinant(s) expressed on the surface of resting or lectin-activated human alloimmune lymphoid cells. The present study confirms and extends previous findings of LT determinants expressed on lectin-activated human T cells (6). Antigens associated with pre-LT form were detectable by a functional immunosorption assay as cell-surface component(s) present on MLC-sensitized human PBL (Fig. 4). The pre-LT antigenic determinants were present on MLC-lymphoblasts both before and after activation with Con A. This result established that "natural" activation with MLC-pelleted at 100,000 x g for 1 hr. Further experiments are in progress to clarify this point.

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REFERENCES


