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MECHANISMS OF LYMPHOCYTE-MEDIATED CYTOTOXICITY

II. Biochemical and Serologic Identification of a Precursor Lymphotoxin 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 lymphotoxin (pre-LT), and in this precursor form, the α and β antigens exist as masked or cryptic determinants. The pre-LT form in unfractionated supernatants was defined immunologically by neutralization with a polyspecific anti-LT antisera (anti-WS), and its lack of reactivity with anti-α and anti-β 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-α LT antiserum. In addition, the pre-LT cytotoxic activity in unfractionated supernatants treated by various physical or chemical means rendered the cytotoxic activity neutralizable by an anti-α 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 γ 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. α 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 α-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 lymphotoxin antisera (anti-LT)[3] in a specific human alloimmune cytotoxic reaction system *in vitro* (1). The capacity 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 α or β 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-α or anti-β 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 the α and β 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 α or β determinants, may be required to inhibit lymphocyte-mediated cytotoxicity.

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 precuror form in which the α and β-associated antigens are masked. Furthermore, the polyspecific anti-LT antisera 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 (Swartz-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, Na H_2PO_4 0.01 M, pH 7.2). α-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 1×10^5 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

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[3] Abbrevations used in this paper: Anti-WS, anti-whole supernatant; LT, lymphotoxin; NGS, normal goat serum; NRS, normal rabbit serum; PBL, peripheral blood lymphocytes; pre-LT, precursor lymphotoxin.

number. Preparation, specificity, and characterization of the xenogeneic anti-LT sera, and the use of the anti-LT sera in a quantitative LT neutralizing assay have also been described in detail (1, 4, 5). The percentage of neutralization of LT activity was determined by the following formula:

$$\% \text{ Neutralization} = \frac{A - B}{T - B} \times 100.$$

where *A* represents the L-929 cell number after incubation with LT treated with antiserum; *B* represents the cell number after incubation with untreated LT; *T* represents the total cell number employed in the assay.

Mixed lymphocyte culture (MLC) and lectin activation of alloimmune effector lymphocytes. 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 1×10^6 cells/ml in medium containing Con A (Sigma) at 10 μg/ml. These cultures were incubated for 5 to 20 hr at 37°C. This 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 rosette 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 re-rosette formation with SRBC. Lymphocyte-bound SRBC were removed by distilled water lysis. The T-enriched lymphocytes were then cultured at 1×10^6 cells/ml in medium containing 10 μg/ml phytohemagglutinin-P (PHA, Difco Laboratories, Detroit, MI) for 24 hr.

Gel filtration chromatography. Freshly isolated supernatant fluid from MLC-primed lectin-activated lymphocytes was concentrated 8- to 10-fold by ultrafiltration (Amicon, PM-10). A 2-ml aliquot was then chromatographed 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, 0.1 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). Molecular weight calibration of this column was conducted with the following markers: thyroglobulin (Tg) 669,000 m.w.; human immunoglobulin (IgG) 150,000 m.w.; hemoglobulin (Hb) 64,000 m.w.; phenol red (PR) 354 m.w.

Measurement of membrane-associated LT. 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 cell pellet, mixed, and incubated on ice for 1 hr. The absorbed antisera were cleared of cells by centrifugation (3000 × G, 10 min) and then tested for their capacity to neutralize LT activity on L-929 cells as described.

Direct lymphocyte-mediated cytotoxic assay. The lysis of WI-L2 target cells by MLC-primed human lymphocytes was performed as described (1).

RESULTS

Identification of an immunologic precursor form of human LT (pre-LT) released by MLC-primed lectin-activated lymphocytes. Preliminary observations 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 U) of activity appearing in the culture fluid within 15 to 30 min after addition of the lectin. High levels of cytotoxic activity, 50 to 1,000 U/ml, were obtained after 5 to 10 hr in culture. This finding was in contrast to non-MLC primed lymphocytes, which required up to 3 to 5 days in culture with lectin to obtain similar levels of cytotoxic activity. The antigenic nature of the cytotoxic activity released by MLC-primed lectin-activated human lymphocytes was investigated by testing various xenogeneic anti-human LT sera for their capacity to neutralize the cytotoxin(s) obtained under these conditions. The results of these experiments are presented in Figure 1. The polyspecific anti-WS was found to be the *only* anti-LT sera capable of completely neutralizing the cytotoxic activity obtained from these cells. A rabbit anti-α_2-LT sera was capable of neutralizing only a small percentage, generally less than 10%, of the activity, even though both antisera had similar neutralizing titers (1). In addition, anti-β-LT sera showed no significant capacity to neutralize the cytotoxic activity in unfractionated supernatants. These results indicated the cytotoxic activity in these supernatants released by alloimmune cells was antigenically distinct from the α and β components of the LT system. Although not shown, similar

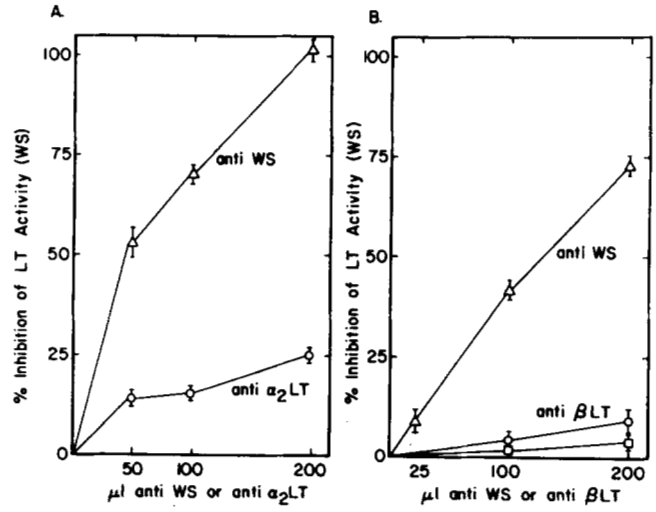

Figure 1. The capacity of rabbit anti-LT sera to neutralize the cytotoxic activity obtained from MLC-primed lectin-activated human lymphocytes. *A*, various amounts of rabbit anti-WS or anti-α_2 LT were tested for their capacity to neutralize 8 units of cytotoxic activity present in freshly isolated whole supernatants (WS) from 5 day MLC-primed 15 hr Con A-stimulated PBL. Cytotoxic activity was measured on L-929 target cells as described in *Materials and Methods*. *B*, whole supernatant from 6 day MLC-primed 20 hr Con A-activated PBL containing 11 units were neutralized by rabbit anti-WS (△——△); anti-β_1 LT (○——○); anti-β_2 LT (□——□). Data represent the mean ± range of duplicate samples. The per cent inhibition of LT activity was determined relative to NRS-treated supernatant activity.

results were obtained with the goat anti-WS or anti-α_2 LT antisera 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 quantitative capacity of the anti-α or β LT sera was not exceeded. In addition, these anti-α and anti-β-LT antisera when tested on whole supernatants from 3- to 5-day lectin-stimulated lymphocytes could significantly neutralize (25 to 80%) a similar amount of LT activity (4, 5). Thus, the failure of the anti-α or β LT antisera to inhibit the cytotoxic activity released from the MLC-primed lectin-activated lymphocytes was a qualitative reaction and indicated the α and β-associated antigens were either masked or not present in these culture fluids.

Relationship of the classically defined components of the human LT system to the pre-LT form. Gel filtration chromatography of the cytotoxic activity present in whole supernatant from MLC-primed lectin-activated lymphocytes revealed that the cytotoxic activity eluted as multiple components with m.w. characteristics similar to the LT-Cx, α, β,and γ LT classes (Fig. 2). The area of the cytotoxic activity peaks revealed the α and β classes comprised the majority of the cytotoxic activity. Recovery of the cytotoxic activity applied to the column was estimated to be 75–85%, indicating that no selective loss of any cytotoxic components had occurred. In contrast to the cytotoxic activity in unfractionated supernatants, additional experiments revealed that the fractionated cytotoxic components were neutralized by the anti-α_2LT antisera. These results are presented in Table I. The nonneutralizable portion of the cytotoxic activity in the β LT m.w. class most likely represents the non-α LT cross-reacting β_1-LT subclass (4, 7). The instability of the cytotoxic activity of the γ LT class prohibited testing of this fraction. Control experiments in which whole supernatant cytotoxic activity was dialyzed against the column buffer (0.01 M phosphate, 10^{-4} M EDTA, 0.15 M NaCl) showed no change in the antigenic properties of these cytotoxin(s) and no decrease in the level of cytotoxic activity was observed when compared to untreated freshly isolated whole supernatants. Passage of the unfractionated whole supernatant over goat anti-α_2 LT IgG covalently linked to Sepahrose 4B did not retain the cytotoxic activity found in these superantants (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

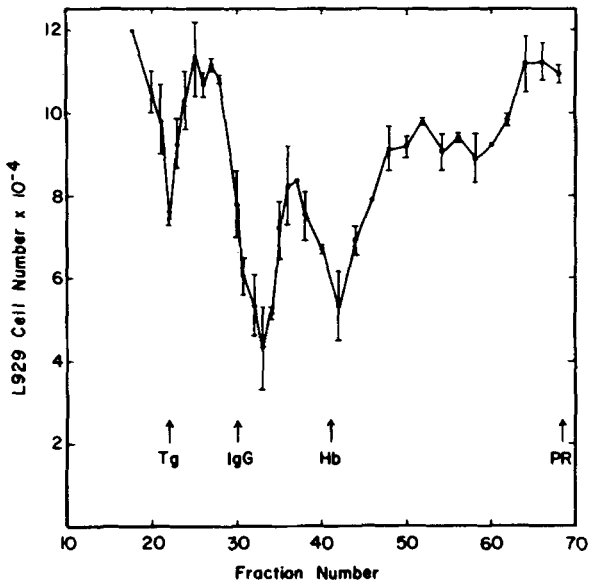

Figure 2. Gel filtration profile of cytotoxic activity from MLC-primed lectin-activated human lymphocytes. Seven day MLC-primed 20 hr Con A-activated PBL supernatant, concentrated 8-fold by ultrafiltration, was chromatographed on a 2.5 x 55 cm column containing Ultrogel AcA 44, as described in *Materials and Methods.*

TABLE I

The capacity of rabbit anti-LT to neutralize the cytotoxic activity of pre-LT after fractionation by gel filtration chromatography[a]

Antiserum Tested	% Neutralization		
	LT-Cx region (Fx No. 22)	α region (Fx No. 34)	β region (Fx No. 44)
NRS	2	0	4
Anti-WS	95 ± 7	97 ± 4	102 ± 6
Anti-α_2 LT	80 ± 2	100 ± 10	47 ± 12

[a] 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 microliters (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_2 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.

the LT activity released by MLC lymphoblasts existed initially as an antigenic precursor form, tentatively designated pre-LT, of the other components in the LT system.

Conversion of the pre-LT to α LT determinants by physical and chemical treatments. Studies were conducted to test the capacity of various physical or chemical treatments to alter the antigenic structure of the pre-LT form present in unfractionated whole supernatants obtained from lectin-activated MLC lymphoblasts and lectin-activated T cells. Alteration of the antigenic composition of pre-LT by various treatments was monitored by measuring the effectiveness of anti-α_2 LT serum to neutralize the cytotoxic activity of these preparations on L-929 target cells. Whole supernatants were treated with high concentrations of potassium chloride, urea, nonionic detergent NP-40, or freeze thawing. One-hundred-microliter aliquots of whole supernatant from MLC-primed Con A-activated PBL or lectin-activated enriched T lymphocytes were treated with these reagents for 30 min. These preparations were then diluted 10-fold with PBS containing 200 μl of either rabbit anti-α_2 LT, anti-WS, or control normal serum. The antisera treated preparations were dialyzed against PBS to remove the chemicals, and the cytotoxic activity was then assayed on L-929 target cells as described in *Materials and Methods.* The results of this experiment are presented in Table II. The data indicate that treatment of pre-LT containing supernatants with urea or detergent signficantly increased the capacity of anti-α_2 LT sera to neutralize the cytotoxic activity present in these supernatants. In contrast, 2 M KCl showed no capacity to alter the antigenicity of this preparation. Freeze-thawing of the supernatant also partially converted pre-LT to the

TABLE II

Conversion of pre-LT obtained from human T or alloimmune lymphocytes to α-LT antigens by various physical or chemical treatments[a]

LT Pretreated with	A. LT Activity (units/ml) of T Cell Pre-LT Activity after Treatment with		
	NRS	Anti-α_2 LT	Anti-WS
Untreated	26 ± 2	21 ± 3	<1
NP-40 (0.1%)	25 ± 1	1	<1
Urea (2 M)	24 ± 5	<1	3 ± 2
KCl (2 M)	27 ± 3	20 ± 1	<1
	B. LT Activity (units/ml) of MLC-Blast Pre-LT Activity after Treatment with		
	NRS	Anti-α_2 LT	Anti-WS
Untreated	33 ± 6	30 ± 2	<1
NP-40 (0.5%)	15 ± 2	1	1
KCl (2 M)	35 ± 3	36 ± 4	1
Urea (8 M)	4 ± 2	<1	<1
Freeze-thaw	32 ± 2	19 ± 4	4 ± 3

[a] One hundred microliter aliquots of supernatants from: A, 24-hr PHA-P-activated T cell-enriched human PBL, or B, 5 day MLC-primed 15 hr Con A-activated human PBL were treated with: 10 μl of 1% or 5% NP-40 in PBS; solid KCl or urea for 30 min at 25°C. Treated supernatants were then diluted 10-fold with PBS containing 200 μl of the indicated anti-LT or normal rabbit sera. These preparations were then dialyzed for 15 hr against 2000 volumes of PBS. Antisera-treated or -untreated (dialysis only) supernatants were then titered for LT activity on L-929 target cells. Untreated samples contained: 28 ± 3 units in A, and 33 ± 4 units in B. Freeze-thawing of supernatants was done once at −20°C for 24 hr (dialysis step was omitted). Data represent the mean ± range of duplicate samples.

α LT antigenic 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, β, and γ classes were all 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 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(s).

Detection of pre-LT and α-LT antigens on the cell surface of alloimmune human lymphocytes. Our previous work had demonstrated that antigenic determinants associated with α LT activity can be detected on the cell surface of nonimmune T lymphocytes after activation with lectins, PHA, or Con A (6). Our next experiments were directed at determining whether the antigenic determinant(s) associated with pre-LT and α LT were detectable on the cell surface of activated alloimmune effector lymphocyte populations. Human PBL were stimulated in one-way MLC against WI-L2

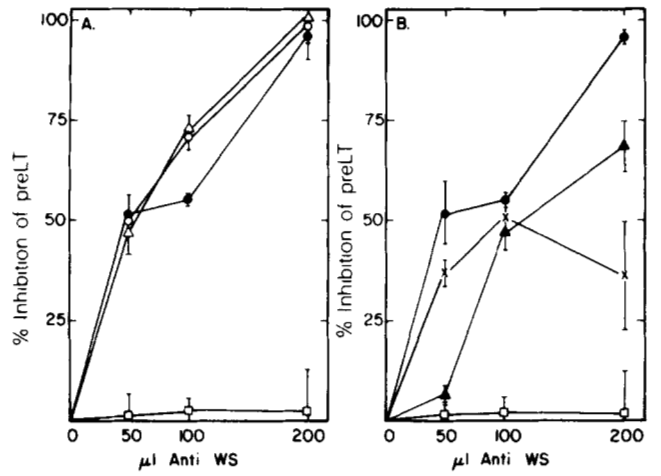

Figure 3. Immunoabsorption of the pre-LT neutralizing activity of rabbit anti-WS with antigens co-fractionating with various m.w. classes of human LT. Rabbit anti-WS was absorbed with 10 to 15 units of the indicated m.w. class of LT activity obtained from MLC-primed Con A-activated PBL. These mixtures were then tested for their capacity to neutralize an additional 5 units of unfractionated supernatant (pre-LT). See *Results* section for further details. *A*, rabbit anti-WS absorbed with: α-LT (○——○); β-LT (△——△); unfractionated WS (□——□); PBS (●——●). *B*, rabbit anti-WS absorbed with: LT-Cx (▲——▲); γ-LT (×——×); unfractionated WS (□——□); PBS (●——●). The per cent inhibition of LT activity was calculated as described in *Materials and Methods*. Data represent the mean ± range of duplicate samples.

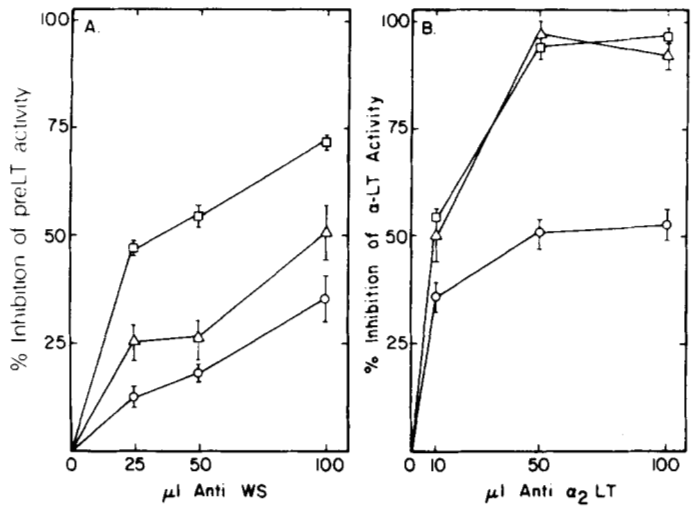

Figure 4. Identification of pre-LT and α-LT associated antigenic determinants present on the cell surface of alloimmune human lymphocytes. Rabbit anti-WS or anti-α_2 LT antiserum was absorbed with either Con A-activated or nonactivated MLC-primed PBL (1.5×10^8 cells/ml antiserum). These absorbed or unabsorbed antisera were then tested for their capacity to neutralize pre-LT or α-LT activity. *A*, rabbit anti-WS serum absorbed with: activated lymphocytes (○——○); nonactivated lymphocytes (△——△); or unabsorbed (□——□); and tested against unfractionated supernatant pre-LT activity from MLC-primed Con A-activated human PBL. *B*, rabbit anti-α_2 LT serum absorbed with: activated lymphocytes (○——○); nonactivated lymphocytes (△——△); or unabsorbed (□——□); and measured on α-LT preparation from lectin stimulated lymphocytes. The per cent inhibition was calculated as described in *Materials and Methods*. Data represent the mean ± range of duplicate samples.

lymphoblastoid 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-α_2 LT antiserum. The absorbed or, as control, unabsorbed antisera were then tested for their capacity to neutralize pre-LT in unfractionated 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-α_2 LT antisera were significantly diminished in their anti-LT neutralizing activities when absorbed on Con A-activated alloimmune lymphocytes. The anti-α_2 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-α_2 LT antiserum and complement (C) to inhibit direct lymphocyte-mediated cytotoxicity of allogeneic WI-L2 target cells. The previous results indicated that α LT-associated antigens were detectable on the cell surface of these MLC-primed lectin-activated lymphocyte populations. The expression of α 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-α LT plus C. Lectin-activated or nonlectin-activated alloimmune lymphocytes were treated with rabbit or goat anti-α_2 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 ^{51}Cr-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-α_2 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-α_2 LT and C. However, rabbit anti-α_2 LT partially inhibited *activated* lymphocytes without the addition of C. These results indicated that an α LT-like determinant(s) was expressed on the activated alloimmune killer cell surface.

Detection of pre-LT and α-LT associated antigens produced during the alloimmune cytotoxic reaction. Goat anti-WS or rabbit anti-α_2 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 ^{51}Cr-release assay. In a parallel ^{51}Cr-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-α_2 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 unfractionated 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 antigens expressed 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." The

TABLE III

Decrease in the cytolytic activity of activated alloimmune lymphocytes by treatment with anti-α_2 LT and C[a]

Expt. No.	Ly:TCG Ratio	% Lysis of WI-L2 after Treatment of Effector with					
		Nonactivated			Lectin Activated		
		NGS + C	Anti-α_2 LT	Anti-α_2 LT + C	NGS + C	Anti-α_2 LT	Anti-α_2 LT + C
1	12:1	37.0 ± 0.2	32.5 ± 6.0	31.3 ± 0.5	34.2 ± 4.0	36.4 ± 0.2	27.6 ± 0.4
	6:1	27.4 ± 2.2	31.3 ± 1.6	24.5 ± 3.0	29.7 ± 1.3	29.7 ± 2.0	19.6 ± 0.2
		NRS + C	Anti-α_2 LT	Anti-α_2 LT + C	NRS + C	Anti-α_2 LT	Anti-α_2 LT + C
2	20:1	19.2 ± 2.0	23.0 ± 1.0	21.6 ± 3.0	22 ± 1.7	16.4 ± 1.1	12.6 ± 1.3
	10:1	12.7 ± 0.6	14.9 ± 1.2	11.6 ± 0.1	13.2 ± 0.7	10.9 ± 1.2	7.9 ± 1.1

[a] Human PBL were sensitized to WI-L2 in MLC for 5 days. Effector cells were then cultured for 15 hr with or without Con A (10 μg/ml). These lymphocytes (2 × 10^6 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-α_2 LT or normal sera. 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 10^4 ^{51}Cr-labeled WI-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.

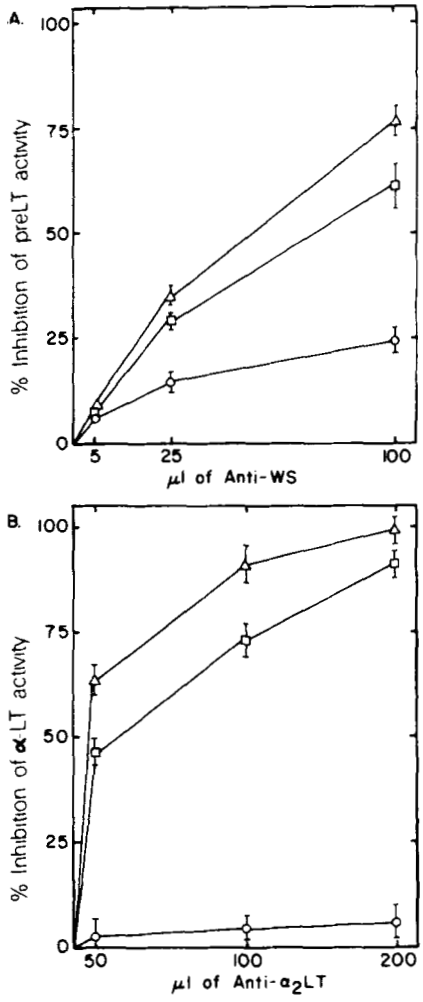

Figure 5. Loss of LT neutralizing activity of anti-WS or anti-α_2 LT after incubation in the human alloimmune cytotoxic reaction. *A*, MLC-generated effector cells (4 × 10^6 cells) were incubated alone or with WI-L2 target cells (25:1 ratio) in the presence of a 1:4 dilution of goat anti-WS for 6 hr 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 unfractionated 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-α_2 LT serum (1:3 dilution). The capacity to neutralize LT activity by this antiserum was measured with 2 units of α-LT that was partially purified by gel filtration chromatography as described in *Materials and Methods*. Anti-α_2 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 polyspecific 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 antiserum 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) Immunoabsorption of the anti-WS with various m.w. LT classes obtained by gel filtration of pre-LT activity revealed the antigenic determinants 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_2 LT antisera (Table II). These findings-established a relationship between the pre-LT supernatant forms 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 each other to form complexes, and the complexes may further associate with nonclassical antigen-binding receptors (11). The relationship of pre-LT, as it exists in the unfractionated 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 (13) 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).[4] In addition, those studies revealed receptor-LT forms are associated with the LT-Cx class and another form, termed $\alpha_H$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_H, 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 × 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 LT activity released by MLC-sensitized lymphocytes upon co-culture with lectin is primarily detecting a T cell response. The reasons to support this conclusion are based on previous findings that prestimulated cells accumulate high levels of intracellular LT pools and rapidly release this material upon restimulation (18, 19), whereas, nonprestimulated cells lack these pools and release much more slowly *in vitro*. Thus, alloantigenic 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 lymphotoxin-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 α determinants expressed on lectin-activated human T cells (6). Antigens associated with pre-LT form were detectable by a functional immunoabsorption 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 allogeneic cells promoted expression of these determinants, and they are not just expressed as a result of Con A activation. It is not yet known if the pre-LT antigenic determinants are present on the cell surface of freshly isolated T lymphocytes or other cell populations. In contrast, α LT-associated antigenic determinants were only detectable as T cell-surface components after lectin activation. Evidence that the α LT component was present on the actual alloimmune effector T cell was shown by the capacity of the anti-α_2 LT antisera and C to abrogate partially the cytolytic activity of this cell population (Table III). The inability of the anti-α LT antisera and C to abolish completely the specific lytic activity of this cell population suggests that the effector cells expressing α LT determinants are heterogeneous. This may reflect an asyncronous rate of activation by lectin to induce the expression of the α LT determinants on the lymphocyte surface. It was shown in the preceding manuscript (1) that concurrent incubation of anti-α LT antiserum in this cytotoxic reaction system failed to inhibit target cell lysis. Thus, lectin activation, followed by treatment of the effector cells with anti-α_2 antiserum and C, is a necessary requirement to allow this anti-LT serum to mediate an inhibitory effect. Collectively, these studies demonstrate that alloimmune cytotoxic effector cells express antigenic determinants associated with cytotoxic molecules of the LT system on their outer membrane surface. Further studies may reveal that the expression of membrane-associated LT molecules may be followed as specific markers of cytotoxic effector cell function and differentiation.

The observation that LT neutralizing activity of anti-WS and anti-α_2 LT sera were diminished after incubation in the lymphocyte-induced cytotoxic reaction demonstrated that pre-LT and α LT molecules are produced in this reaction after contact between the effector and target cells (Fig. 5). The lack of significant levels of detectable LT activity in the supernatant of this cytotoxic reaction suggests that LT molecules were either not freely released into the supernatant or the biologic activity of the LT molecules was rapidly lost under these conditions. The latter possibility seems unlikely, since α LT-associated activity is quite stable (10). This result further implies a restriction on the mechanism of LT action in this system, i.e., the LT effector molecules may be restricted to membrane contact regions. The free diffusion of these cytotoxins would most likely be limited by their association with the antigen receptor.

The components associated with LT activity must be viewed in a different perspective, since they appear to form an interrelated system of cell-lytic molecules (4, 8, 9). The situation is complex, since there are physical and immunologic differences between the various forms and certain of the larger forms are associated with nonclassical antigen receptors (11) and possess increased cell-lytic activity (12). The results presented here have applicability to the interpretations given to the results on the effects of various anti-LT sera in the alloimmune cytotoxic reaction (1). First, an additional antigenic component(s), pre-LT, has tentatively been defined, and thus adds further to the immunologic heterogeneity exhibited by human LT. The first form of LT released by the activated effector cell appears to represent an antigenic precursor of the smaller α, β, and γ components. The antigenic determinants associated with the pre-LT form, in part, reside on the LT-Cx and γ LT molecules. However, additional studies will be required to identify possible neoantigens expressed by the pre-LT form. We feel that the inhibitory action of the anti-WS in the T cell cytotoxic reaction is mediated largely by the capacity of the anti-WS to react collectively with antigens associated with several determinants of the LT system including the pre-LT form. Although alloimmune effector cells expressed the pre-LT form on their surface, preabsorption of these effector cells with the anti-WS did not significantly block their capacity to lyse target cells (Table VII, Reference 1). This result suggests anti-pre-LT antibodies alone were not sufficient to block lysis, indicating contributions from additional antibody specificities were required to inhibit cell killing. This contention is further supported by our findings that activated lymphocytes were more efficient than nonactivated lymphocytes in absorbing the inhibitory activity of the anti-WS (Table VI, Reference 1). Alternatively, the pre-LT-antibody complexes may have been released or shed from the effector cell surface rapidly being replaced by lytically active forms of pre-LT that mediates target cell lysis. The question is addressed further in the following manuscript (19). Second, the finding that α and β LT-associated antigens are masked or at least inaccessible to neutralizing antibodies in the pre-LT form may, in part, explain why the individual anti-α or β-antisera failed to inhibit this alloimmune cytotoxic reaction. However, the loss of α LT-neutralizing activity of the anti-α_2 LT serum after incubation in the alloimmune cytotoxic reaction (Fig. 5) indicates that at least some α LT determinants were accessible to antibody. It is difficult to interpret these results, since we do not presently know at what time or place during the cytotoxic reaction that α determinant(s) become accessible to antibody. For instance, if the α determinant is exposed only after delivery to the target cell, it may be too late for an antibody to reverse the inflicted damage, although the antibody may still bind to the determinant(s). Alternatively, some α LT determinants may represent degradation products of the larger lytically active forms. Further study will be required to clarify this situation.

The studies presented here provide additional evidence supporting the concept that components of the LT system play an important role in the lytic effector mechanism employed by human alloimmune cytotoxic effector cells. Studies presented in the third article of this series expand the present investigations on the mechanism of the inhibitory action of the anti-WS in the human alloimmune cytotoxic reaction and describe the usefulness of these

[4] Ware, C. F., J. Klostergaard, M. K. Toth, R. S. Yamamoto, and G. A. Granger. 1980. The Human LT System. IX. Serological identification of $F(ab')_2$, alloantigens, and C1q-like determinants associated with human lymphotoxins (submitted).

reagents in dissecting the lytic effector mechanism of cytotoxic T cells (20).

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