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In Vitro Lymphocyte Cytotoxicity

II. Unstable Lymphotoxins (β-LT) Secreted and Inactivated by Mitogen-Stimulated Human Lymphocytes[1]

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Supernatants from phytohemagglutinin (PHA)-activated human lymphocytes contain two major classes of cytotoxins (α-LT and β-LT). While α-LT appears to be stable, and the major component in 5-day culture supernatants, the majority of cytolytic activity at earlier intervals in these cultures is due to a "family" of highly unstable cytotoxins which are both secreted and destroyed at a rapid rate. The inactivation of the unstable LT molecules appears to be due to: (a) inherent instability of β-LT molecules, and (b) a lymphocyte-mediated inactivation mechanism(s) which involves serum.

INTRODUCTION

When lymphoid cells from experimental animals or humans are stimulated *in vitro* with mitogens or antigens or during mixed lymphocyte culture, they release into the supernatant a variety of biologically active macromolecules, collectively termed lymphokines (LK) (1–4). One family of cytotoxic or cytostatic LK (lymphotoxin (LT)) has been suggested to be important effectors perhaps involved in cellular immune tissue destructive reactions, such as allograft rejection and tumor immunity (4, 5).

Several investigators have reported that the LT activity in supernatants obtained from phytohemagglutinin (PHA)-activated human lymphocyte cultures consists of a multicomponent family of macromolecules (6–11). The two predominant activities, termed α-LT and β-LT by Walker *et al.* (10), have molecular weights of 75–90,000 and 45–50,000 daltons, respectively, based on their Sephadex elution profiles or sedimentation velocity on sucrose gradients (7, 9). As opposed to α-LT, it has been shown that β-LT appears to be relatively unstable (10). The present study was undertaken to investigate further the reasons for this instability.

METHODS

Supernatants from PHA-activated human lymphocytes (SAL) were obtained as described previously (12). Briefly, a suspension of human tonsil or adenoid lympho-

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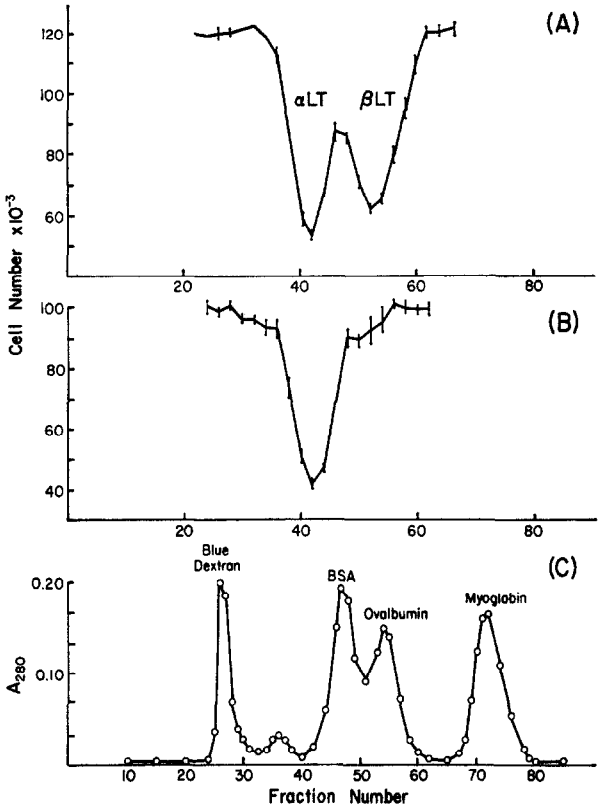

FIG. 1. Stability of cytotoxic LEM secreted by human lymphocytes *in vitro* when stored at 4°C. Supernatants from PHA-stimulated adenoid lymphocytes were collected 24 hr post-stimulation, concentrated 10×, and 1.5 ml were chromatographed on Sephadex G-150. (A) The column fractions were filter sterilized and tested for LT activity. The elution profile shows two peaks of LT activity, termed α-LT and β-LT. (B) Column profile after storage of the same fraction for 8 days at 4°C. Assays in (A) and (B) were performed in an identical manner, and error bars represent cell counts in duplicate tubes. (C) Column calibration showing elution profile of MW markers, blue dextran (2×10^6), bovine serum albumin (68,000), ovalbumin (44,000), and myoglobin (18,000).

cytes was obtained after density gradient centrifugation using Ficoll-Hypaque. These cells were routinely 95–98% viable and 95–98% lymphocytes. Cultures were established at 4×10^6 cells/ml in Hanks' minimal essential medium (MEM), supplemented with either 10% fetal calf serum (FCS) or 20 μg/ml of a heat-stable serum fraction (BS) (13), 100 μg/ml of streptomycin and 100 U/ml of penicillin. Activation of the lymphocytes was effected with PHA (Lot No. 604193, Difco, Detroit, Mich.) at 20 μg/ml. After a 14- to 24-hr incubation at 37°C, the cells were removed by centrifugation at 400*g*, the supernatant was filter sterilized by passage through a 0.45-μm Millipore filter, and dilutions were tested for LT activity on mitomycin C-treated L-cells, as previously described (14). The units of LT activity in a given supernatant are obtained by determining the reciprocal of the dilution killing 50% of the target L-cells. In some experiments, SAL were concentrated by ultrafiltration through an Amicon PM 10 filter, followed by separation on a 2.5 × 100-cm Sephadex G-150 column equilibrated in phosphate-buffered saline, pH 7.2 (PBS). The column fractions obtained were filter sterilized and tested for

LT activity by adding 0.2 ml to a 1-ml monolayer tube culture of 10^5 L-cells. After a 24-hr incubation at 37°C, the remaining adherent cells were enumerated with a Coulter counter.

RESULTS

Instability of β-LT during storage at 4°C. An example of the instability of human β-LT molecule(s) is evident in Figs. 1A and B, in which the Sephadex G-150 elution profile of a concentrated SAL obtained 24 hr after PHA stimulation in MEM + 10% FCS is shown. The elution profile in Fig. 1A shows two peaks of LT activity (α and β). In contrast, Fig. 1B shows the same column fractions assayed in an identical manner after storage for 8 days at 4°C. It is clear that the β-LT activity is unstable under these conditions.

Effect of standard culture conditions on the stability of β-LT. Experiments were next initiated to examine the stability of the LT molecules under standard conditions of culture. SAL from PHA-stimulated human lymphocytes maintained in MEM supplemented with 10% FCS were collected after 14 hr. The cells were removed by centrifugation, the media were filter sterilized and either immediately assayed for LT activity or tested after incubation at 37°C for various intervals. The results of a representative experiment of four similar experiments can be seen in Fig. 2. SAL tested immediately after removal from culture had high levels of toxic activity but, when incubated at 37°C, rapidly lost activity with an apparent half-life of 2–3 hr. The activity remaining after 9 hr of incubation at 37°C was shown

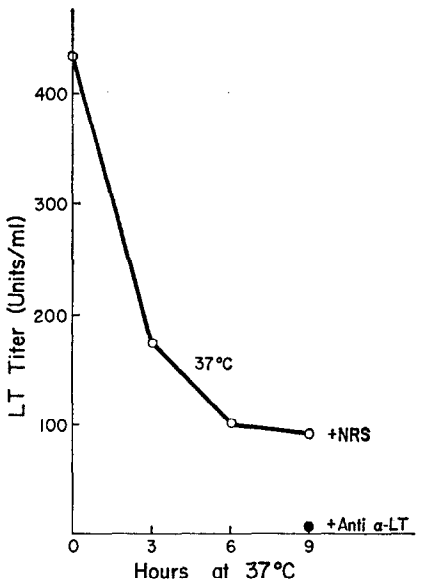

FIG. 2. Inactivation of cytotoxic LEM at 37°C. Human adenoid lymphocytes were stimulated with 20 μg/ml of PHA-P in MEM supplemented with 10% FCS and the SAL collected after 14 hr. The SAL were filter sterilized and immediately assayed for LT activity (0 time point) or incubated at 37°C for various periods of time and then assayed. At 9 hr, rabbit anti-human α-LT or normal rabbit serum (0.1 ml/1 ml of SAL) was added just before assaying for LT activity. All results are expressed as the average of duplicate determinations. Standard error is consistently less than 15%.

TABLE 1

Stability of the α-LT Family of Human Cytotoxic LEM at 37 and 56°C

Days incubated	5-Day SAL[a] 37°C	α-LT (Sephadex fractions)[b]	
		37°C	56°C
0	240 ± 20	63 ± 5	63 ± 5
1	180 ± 10	65 ± 5	60 ± 5
2	—	—	55 ± 3
5	180 ± 15	58 ± 8	—

[a] Crude SAL was obtained 5 days after PHA stimulation of lymphoid cells from adenoid tissue and stored at −20°C until used. Samples to be tested were incubated at 37°C for various periods of time and then stored at −70°C. One sample was placed at −70°C, at the onset of the experiment, as a Day 0 control. At the end of 5 days, all samples were then tested for LT activity as described in Methods.

[b] The fractions containing α-LT activity were obtained from a 48-hr SAL, which was concentrated and chromatographed as described. The fractions containing α-LT activity were tested for stability at 37 and 56°C, identical to the method used with SAL. Results are expressed as the average of duplicate points ± standard error.

to be due to α-LT, since it was completely neutralized by treatment with rabbit anti-human α-LT sera (15). The stability of α-LT was further supported by the finding that this material obtained from Sephadex fractions or present in 5-day cultures could be heated at 56 or 37°C for several days with very little loss of activity (Table 1).

Identification of a lymphocyte-serum-dependent inactivation mechanism. During the initial phases of these studies, we noticed a relationship between instability of cytotoxic activity in SAL or column fractions and the presence of serum components. To investigate this observation further, lymphocyte cultures were established in MEM, supplemented with either 10% FCS or 20 μg/ml of BS and 20 μg/ml of PHA-P. After 14 hr of incubation at 37°C, the SAL from both cultures were either immediately assayed or incubated at 37°C for various periods of time, and then tested for LT activity. The result of one experiment from these studies is shown in Fig. 3. The LT activity in SAL from cultures maintained in 10% FCS decays rapidly (Fig. 3, left) and reaches a plateau after approximately 9 hr. The remaining activity after 9 hr is due to α-LT, since anti-α-LT antibody neutralized only 50% of the total LT activity at time 0 (indicated by dashed line). However, LT activity in SAL from cultures maintained in BS is more stable (Fig. 3, right) and decays only slightly after 9 hr. Furthermore, in several experiments, the addition of 10% FCS to the lymphocyte-free SAL containing BS (BS + 10% FCS) did not increase the inactivation rate. In addition, the level of α-LT (indicated by dashed line) in the SAL produced in BS was also determined by neutralization of the α-LT activity by anti-α-LT antibody at time 0.

DISCUSSION

This report further supports the existence of two major classes of cytotoxic LEM released *in vitro* by PHA-activated human lymphoid cells. The first class consists of a set of molecules which have a half-life as short as a few hours at 37°C in

cultures containing serum. These unstable molecules appear to belong to the β-LT (50,000 dalton) class of cytotoxins. This situation is complex, however, since (a) there are multiple members of this family, and (b) there appear to be several different reasons for their instability. Recent studies have shown that β-LT can be fractionated into two distinct components by chromatography on DEAE-cellulose and electrophoresis on polyacrylamide gels (16). We found that one of these components (termed β-LT_1) is inherently very unstable at any temperature, regardless of the presence or absence of serum. The instability of the other component, β-LT_2, is dependent on the presence of serum in culture. Purified fractions of β-LT which are serum free are quite stable even to heating at 56°C for several hours. Furthermore, the present results suggest that the instability of this molecule in culture requires the presence of both the lymphocyte and serum together to explain the inactivation. This conclusion is based on the following observations: (i) β-LT activities are quite stable in supernatants produced in cultures supplemented with boiled serum; (ii) β-LT activity in serum-containing medium is more stable when separated from serum components by physical-chemical methods; (iii) finally, adding serum to an active lymphocyte-free supernatant prepared in BS did not increase the rate of degradation. These data suggest a requirement for the presence of both the lymphocyte and serum together with two possible mechanisms to explain the inactivation: (a) The lymphocyte produces an inactivating LEM in the presence of serum, or (b) the lymphocyte activates a serum component to inactivate the cytotoxin(s).

Since the α-LT class of cytotoxins is quite stable, these cytotoxins are the best characterized and are considered by most investigators to be the major components present in lymphocyte supernatants. This may be a mistaken concept, because of the

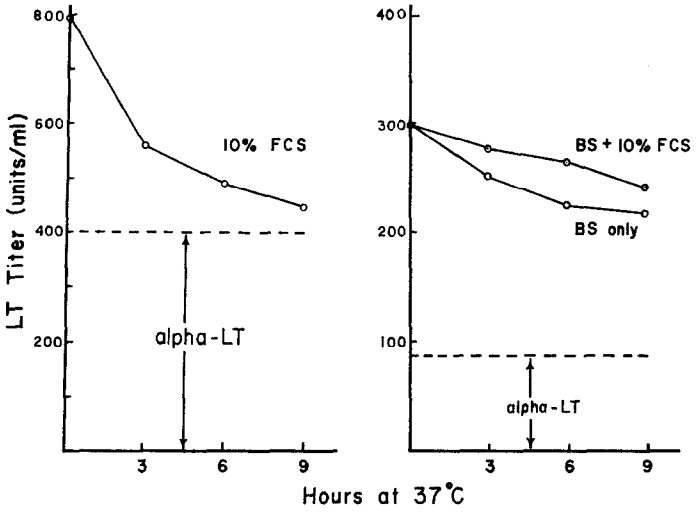

FIG. 3. Relative stability of cytotoxic LEM in SAL produced with serum or serum substitutes. Human adenoid lymphocytes were activated with PHA in the presence of MEM supplemented with either 10% FCS (left) or 20 μg/ml BS (serum substitutes) (right). The SAL were collected 14 hr poststimulation and assayed as described in Fig. 2. To one set of SAL (those produced in BS), FCS was added to 10% (BS + 10% FCS) prior to the 37°C incubation. Dashed lines represent the amount of α-LT in each SAL as determined by neutralization of the α-LT activity with rabbit anti-α-LT antisera (100 μl/ml of SAL).

half-life of the unstable cytotoxins, which may, in fact, be the major cytotoxins elaborated *in vitro* by mitogen-activated human lymphoid cells.

It is interesting to speculate about the role of these molecules in tissue destructive CMI reactions. Preliminary evidence indicates that when the unstable LT molecules are bound to the surface of a target cell, they become stabilized, and, when free, they are rapidly inactivated. It is possible that when an effector lymphocyte contacts a target cell, it becomes activated and secretes the cytotoxins; some bind to the target cell plasma membrane and become stabilized, while the unbound molecules are rapidly inactivated. Thus, these nonspecific LEM would be restricted to short ranges and functionally behave as specific cytotoxins only effective in the microenvironment at the junction regions between aggressor and target cells.

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