Title

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Journal

Molecular Immunology, 18(12)

ISSN

0161-5890

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Publication Date

1981-12-01

DOI

10.1016/0161-5890(81)90020-1

Molecular Immunology, Vol. 18, No. 12, pp. 1049–1054, 1981.
Printed in Great Britain.

0161–5890/81/121049–06$02.00/0

PURIFICATION OF HUMAN ALPHA-LIGHT CLASS LYMPHOTOXIN TO ELECTROPHORETIC HOMOGENEITY

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(*First received* 15 *December* 1980; *accepted in revised form* 24 *March* 1981)

Abstract—We have purified to homogeneity the α_L-component (70,000–90,000) of the human LT* cytolytic system. This lymphokine was purified ~ 10,000-fold from supernatants of lectin-stimulated human tonsil and adenoid lymphocytes by molecular sieving, ion-exchange chromatography on DEAE-Sepharose, and preparative PAGE. The homogeneity of the radiolabeled molecule was confirmed both by electrophoresis and electrofocusing. The identity of the labeled peak with the lytic activity was demonstrated with a concomitant bioassay of the electrophoresed preparation; in addition, immunoprecipitation with a heterologous specific anti-α_L antiserum showed simultaneous precipitation of the radiolabeled component and lytic activity. Immunological and biochemical evidence has previously shown this molecule to be a subunit of the Cx- and α_H-LT forms. The latter LT classes are of intense interest because of their capacity for rapid selective cell lysis.

INTRODUCTION

LT was originally described as a weak, nonspecific growth-inhibitory or cell-lytic lymphokine (Ruddle & Waksman, 1968; Granger & Williams, 1968; Jeffes & Granger, 1975). Recent functional and biochemical studies on LT from human and animal species indicate that LT is in fact represented by a family of diverse mol. wt cytotoxins (Walker *et al.*, 1976; Granger *et al.*, 1978; Ross *et al.*, 1978). The lower mol. wt forms (alpha-light, 70,000–90,000 d; beta, 35,000–50,000 d; gamma, 12,000–20,000 d) are capable only of protracted lysis of a limited number of continuous cell lines; the larger forms (complex, >200,000 d; alpha-heavy, 120,000–160,000 d) are capable of causing rapid nonspecific lysis of a spectrum of allogeneic cells, and selective lysis when obtained from lectin-stimulated alloimmune immune lymphocytes (Yamamoto *et al.*, 1979; Hiserodt *et al.*, 1978*a*). Both immunological and biochemical evidence suggests that these classes are comprised of related subunits (Yamamoto *et al.*, 1978; Hiserodt *et al.*, 1978*b*).

The definitive assignment of a role for these mediators in cell-mediated immune reactions *in vitro* is as yet not possible. The most suggestive evidence for their involvement is derived from serological studies conducted in several laboratories (Walker & Lucas, 1973; Gately *et al.*, 1976; Hiserodt & Granger, 1977; Sawada & Osawa, 1978; Ware & Granger, 1981). It is evident from these studies that antisera which recognize determinants expressed by these soluble toxins may block several types of cell-mediated cytotoxic reactions *in vitro*. Nevertheless, an analysis of the participation of these mediators in the molecular mechanisms of cell-mediated cytotoxicity will be greatly facilitated by the availability of these cytotoxins in a homogeneous form.

The purification of LT has been very difficult, since these mediators are present in extremely low concentrations in lymphocyte supernatants. We have employed serum-free culture conditions to greatly reduce the amount and complexity of exogeneous protein (Lewis *et al.*, 1976); furthermore, we have been able to monitor protein in the picomole range utilizing a mild radiolabeling technique (Klostergaard *et al.*, 1980). As a result, we have been able to purify to electrophoretic homogeneity the human α_{L2}-subclass, an important subunit of the Complex and alpha-heavy classes.

MATERIALS AND METHODS

The methods involved in the preparation of human lymphocyte supernatants, in generating the various mol. wt LT classes by molecular sieving of the supernatants, and in resolution of the α_L-LT class into its charge subclasses by ion-exchange chromatography have been published previously in detail (Granger *et al.*, 1978; Lewis *et al.*, 1976) and will only be briefly summarized here.

* Abbreviations: α_L-LT, alpha-light class lymphotoxin (70,000–90,000 d); α_H-LT, alpha-heavy class lymphotoxin (120,000–160,000 d); α_{L2}-LT, alpha-light class, subclass 2 lymphotoxin; Cx-LT, Complex lymphotoxin (>200,000 d); LT, lymphotoxin; PAGE, polyacrylamide gel electrophoresis.

Table 1. Estimated specific activity and yield of human α_{L2}-LT subclass during purification

	Specific activity[a] (units/microgram of protein)	Increase compared to whole supernatant	Approximate yield (%)
Whole supernatant	< 1	—	
Molecular sieving—α-class	~5	12–15	~70[b]
Ion-exchange chromatography —α_2-subclass	~25	~75	~35[c]
Preparative electrophoresis	~2500	~7500	~10–15
Analytical electrophoresis	~5000	~15,000	

[a] Determined by the fluorescamine assay, using a BSA standard (Klostergaard *et al.*, 1980).
[b] α-Class is the principal form in the supernatant.
[c] α_2-Subclass is the major component in the α-class.

Supernatants from human tonsil and adenoid lymphocytes stimulated with PHA-P (Difco Laboratories, Detroit, MI), were obtained and concentrated as previously described (Lewis *et al.*, 1976). The 5-day cultures were supported by a heat-stable fraction of bovine serum; this novel serum substitute reduces the amount of exogenous protein in the supernatant by about two orders of magnitude. Concentrated supernatants were subjected to molecular sieving on Ultrogel AcA 44 to resolve the various LT mol. wt classes. The α_L-class (70,000–90,000 d) was thereby separated from much of the contaminating protein (Table 1). The α_L-subclasses were obtained from the α_L-class by ion-exchange chromatography on DEAE-Sepharose. The predominant activity, the α_{L2}-subclass (Table 1), which was eluted on a salt gradient, was then subjected to a variety of purification schemes.

Radioiodination of lymphocyte supernatants and LT preparations with ^{125}I (New England Nuclear, Boston, MA) was performed by the Iodogen (Pierce Chemical Co., Rockford, IL) technique as previously described (Klostergaard *et al.*, 1980).

Preparative PAGE of radioiodinated α_{L2}-LT using a discontinuous buffer system was conducted in an apparatus designed by Furlong *et al.* (1973). The preparation was brought to 20% sucrose and then overlayed on a 3% acrylamide stacking gel (1 cm dia, 1 cm high); the separating gel was 7% acrylamide (1 cm dia, 3 cm high). The gel was run at constant current (11 MA) at 4°C. Fractions (20 drops) were collected at a flow rate of 60 drops/hr, employing a Shandon-type apparatus. Fractions were tested for both radioactivity in a Biogamma counter (Beckman Instruments, Fullerton, CA) and for lytic activity as described later.

Analytical PAGE

Fractions containing lytic activity from preparative PAGE of α_{L2}-LT were pooled, concentrated, radioiodinated and subjected to analytical discontinuous PAGE (Davis, 1964). Tube gels (3 mm dia) with a 1 cm stack of 3% acrylamide and a 7 cm running gel of 7% acrylamide were employed. Electrophoresis was conducted at 5 MA per gel at 4°C. After the run, gels were sliced in 2 mm sections and counted in a gamma counter; the slices were allowed to elute overnight at 4°C, in 0.3 ml of media, and then the eluate was assayed on L-cells for lytic activity (see later).

Analytical isoelectricfocusing

Radioactive fractions containing lytic activity from analytical PAGE were subjected to isoelectricfocusing in tube gels. The acrylamide concentration was 4%, and the ampholine gradient was 1% from pH 4 to 8. After running, the gels were sliced (2 mm) and the radioactivity in each slice determined.

Neutralization and immunoprecipitation of ^{125}I-α_{L2}-LT with rabbit anti-human α_{L2}-LT antiserum

Aliquots containing two to four units of radioiodinated α_{L2}-LT (~1000 cpm) from analytical PAGE were incubated with 0–20 μl of a rabbit anti-human α_{L2}-LT antiserum. This antiserum had been raised against electrophoretically purified α_{L2}-LT (Yamamoto *et al.*, 1978). After incubation at room temperature for 30 min, a sheep antiserum (0–20 μl) to rabbit serum was added and the mixture was allowed to incubate for an additional 30 min. The complexes were pelleted by centrifugation. The supernatants were tested on L-cells for lytic activity (see later), and the pellets were counted in a Biogamma counter (Beckman Instruments, Fullerton, CA). As a control, a normal rabbit serum was used in the same manner as the immune serum. Radioactivity precipitated by the immune serum was corrected for the amount

reacting nonspecifically with the normal serum (~15%).

LT assay

The detection and quantitation of LT activity in lymphocyte supernatants, column fractions, and in gel slices was carried out as previously described (Granger *et al.*, 1978). Briefly, 1×10^5 mitomycin-C (Sigma, St. Louis, MO) treated α-L-929 cells were cultured for 24 hr in slant tubes in 1 ml RPMI-1640 with 3% newborn calf serum (GIBCO, Grand Island, NY). Aliquots (10–200 μl) of supernatant, column fractions, or gel eluates were added to the cultures; after 16–24 hr, the remaining viable adherent cells were determined in a Coulter Counter. A unit of killing was defined as that required to decrease the adherent cell count by 50%.

RESULTS

The human α_{L2}-LT subclass was obtained by tandemized molecular sieving and ion-exchange chromatography of supernatants. The α_{L2}-component was first radiolabeled with ^{125}I by the solid-phase Iodogen technique, which preserved lytic activity, so that both protein and lytic activity could be followed in the course of determining optimum purification (Klostergaard *et al.*, 1980).

Both lectin- and hydrophobic-affinity chromatography were initially used independently in purification, since the α_{L2}-component binds to Con A–Sepharose and to decyl-agarose. Despite the fact that 10–20-fold purification could be realized, in either case analysis on PAGE revealed that the adsorbent also retained contaminating proteins resident in the crude α_{L2}-preparation (Klostergaard *et al.*, 1980). It was apparent from these experiments

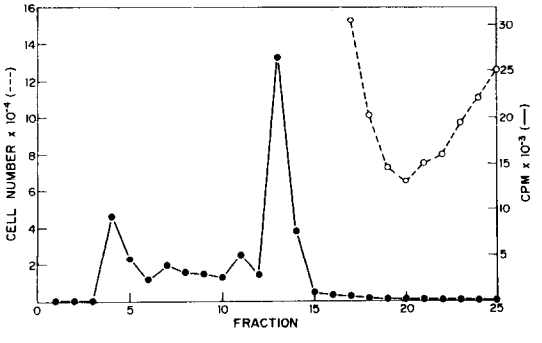

Fig. 1. Preparative discontinuous PAGE (7% acrylamide) of radioiodinated human α_2-LT. Each fraction was tested for lytic activity (O--O) and radioactivity (●—●). Most rapidly electrophoresing components appear in the early fractions.

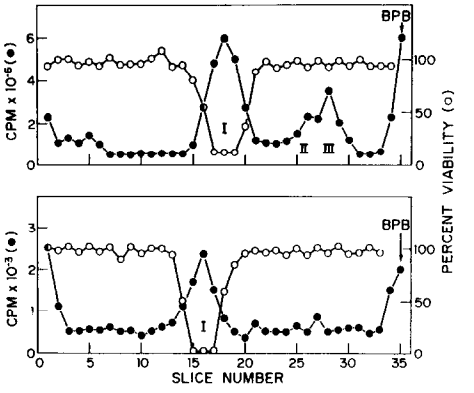

Fig. 2A (top panel). Analytical discontinuous PAGE (7% acrylamide) of human α_{L2}-subclass LT purified by preparative PAGE (fractions 18–23 from Fig. 1). Those peak fractions were pooled, concentrated and radioiodinated. Following analytical PAGE, the radioactivity in each gel slice was determined (●—●). Each slice was eluted in buffer overnight; the eluate was then assayed on L-cells (O—O). B (bottom panel). Analytical discontinuous PAGE (7% acrylamide) of peak I from Fig. 2A. Radioactivity (●—●) and lytic activity (O—O) in each slice was determined as for Fig. 2A. BPB–bromphenyl blue.

that most of the contaminants in these preparations had distinctly higher electrophoretic mobilities than the actual LT moiety; this suggested that a preparative electrophoretic or electrofocusing step might be effective in our purification scheme.

A very striking purification could in fact be achieved by preparative PAGE (Fig. 1). Most of the contaminating proteins migrate rapidly and appear in the early fractions where no lytic activity is present. Lytic activity appears in later fractions which contain very little protein. This electrophoretic step gives an about two orders of magnitude increase in specific activity (Table 1).

In order to obtain α_{L2}-LT from preparative electrophoresis which was of suitable specific activity (radioactivity), fractions from preparative electrophoresis containing the lytic activity were pooled, concentrated about 10-fold on an Amicon PM-10 membrane, and radioiodinated. When analysed on analytical PAGE (Fig. 2A), multiple radioactive peaks are apparent; the peak with the lowest R_f (I) coincides well with lytic activity. The faster moving peaks appear to be trailing peaks from preparative electrophoresis, since they may be substantially reduced when fractions from the lytic peak closest to the contaminating peaks are eliminated from the pool.

Peak I from Fig. 2A appears to be a true electrophoretic component, migrating indistinguishably from lytic activity (Fig. 2B).

Peak I from Fig. 2A was subjected to

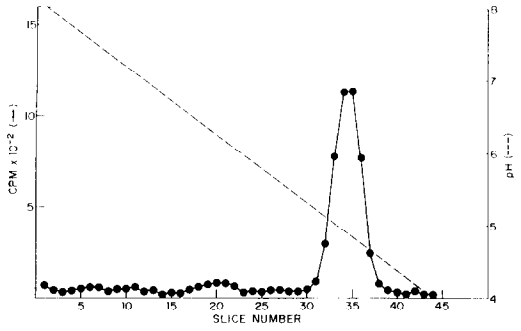

Fig. 3. Analytical isoelectricfocusing in polyacrylamide gels (4% acrylamide) of peak I from Fig. 2A. A 1% ampholine gradient spanned pH 4–8. The radioactivity in each slice was determined (●—●).

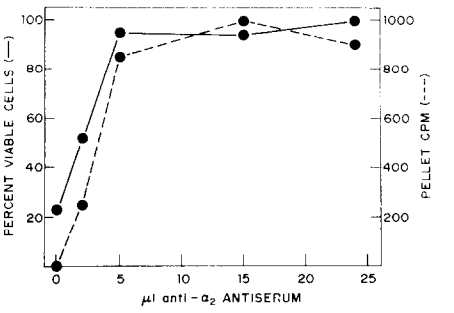

Fig. 4. Blocking of lytic activity and simultaneous immunoprecipitation of radiolabeled α_{L2}-LT. Aliquots of peak I from Fig. 2A were reacted with increasing levels of a rabbit anti-human α_2 antiserum; resulting complexes were precipitated with a sheep antiserum to rabbit serum. Supernatants were tested for lytic activity on LT cells (●—●) and the radioactivity in the pellets was determined (●– –●). The results from each level of immune serum were compared to those for the same level of normal rabbit serum, and the difference plotted.

isoelectricfocusing in polyacrylamide gels. Each gel slice was assayed for radioactivity. Fig. 3 indicates that peak I is extremely homogeneous, with a pI of 4.8.

Aliquots of peak I were reacted with increasing amounts of a rabbit antiserum raised against human α_{L2}-LT which had been purified by native PAGE (Yamamoto *et al.*, 1978). The immune complexes formed were precipitated by the sheep antiserum against rabbit serum; lytic activity remaining in the supernatant was evaluated on L-cells, and the radioactivity in the pellet was determined. The results are seen in Fig. 4.

As the amount of primary antiserum added is increased, lytic activity neutralized and radioactivity immunoprecipitated increases steadily until a level of 5 μl of antiserum is added in the experiment shown here. At this level, all lytic activity is blocked, and radioactivity is totally immunoprecipitated. The coincidence of total blocking and immunoprecipitation of peak I at the same level of added antiserum is strong serological evidence that peak I is homogeneous and identical with the lytic moiety.

DISCUSSION

This report documents the purification to homogeneity of an important member of the human LT family, the α_{L2}-component. This lymphokine is thus one of the very few convincingly purified to homogeneity. The purification scheme surmounted considerable difficulty, since despite beginning with serum-free, low-protein supernatants, it achieved an increase in specific activity of about 10,000-fold.

It is very clear from our data (Table 1, Figs 2–4) that the introduction of radioiodine (^{125}I) into the LT preparation (Klostergaard *et al.*, 1980) was essential for protein detection of sufficient sensitivity to demonstrate homogeneity in gel systems. In particular, two previous reports dealing with the purification of human α_{L2}-LT (Granger *et al.*, 1973; Russel *et al.*, 1972) could not demonstrate a protein moiety truly migrating with lytic activity in PAGE. In fact, Russel *et al.* (1972), using a scheme apparently identical to that in this report, demonstrated in their most purified preparation significant stainable contaminants migrating in PAGE very near to regions containing lytic activity which were not stainable. We believe that their purest LT preparation was probably at least an order of magnitude from homogeneity.

We are convinced that the criteria for purity presented here comprise a very solid basis for the statement that we have in fact purified α_{L2}-LT to homogeneity. First of all, the labeled component is indistinguishable from the lytic moiety in native PAGE, migrating as a single component (Fig. 2A and B). Secondly, the homogeneity of this component is further verified, since it focuses as a sharp peak in isoelectricfocusing (Fig. 3). It is highly unlikely that another component has co-electrophoresed with and has the same pI as the lytic molecule, and that the sharp peaks seen in both electrophoretic systems are due to multiple components. The serological studies further discount this remote possibility. The rabbit antiserum was raised against human α_{L2} which was purified by PAGE. As seen from the scheme presented in this paper (Table 1, Figs 1 and 2A), this is a fairly pure antigen. Even if the antiserum is not entirely monospecific, the fact remains that *exactly* the same levels of antiserum are required to completely neutralize the lytic activity of peak

I (Fig. 2A) as to completely immunoprecipitate the radioactivity in the peak. In totality, the biological, biochemical and serological evidence presented here strongly support the homogeneity of the purified α_{L2}-LT preparation.

Purification of the α_{L2}-LT component has brought certain goals closer to realization. In our own laboratory, we are now examining its peptide subunit composition. These subunits will be compared to the β- and γ-LT classes by immunoprecipitation with the anti-α_{L2} antiserum, and analysis on SDS reducing gels and by peptide mapping. Since we have also recently purified the human receptor-bearing α_H-LT class homogeneity (Klostergaard & Granger, 1981), these peptide maps will be compared to the map of the α_H-form, to examine the subunit relationship of α_L and α_H at this level. In light of the report by Lee & Lucas (1976), we will also test the pure α_L-LT preparation for ribonuclease activity.

In conjunction with other laboratories, we are currently testing the pure α_L- and α_H-LT preparations in other lymphokine assay systems. Provocative preliminary data indicate that a specific anti-human α_{L2}-LT antiserum blocks human monocyte chemotactic factor activity (Yoshida & Granger, unpublished observations). Finally, the possible greater susceptibility of transformed cells, compared to normal cells, to the cytotoxic action of pure lymphotoxins is to be tested in our laboratory. Since a previous report demonstrating high-affinity receptors for LT on sensitive targets and low-affinity receptors on resistant targets was apparently based on the use of an impure LT preparation (Tsoukas *et al.*, 1976), we will reexamine this question with the pure mediator.

Acknowledgements—We wish to thank Mr Robert Yamamoto for the rabbit anti-α_{L2} antiserum, Ms Diane McGriff for the analytical isoelectricfocusing run, and Mrs Gloria Heidenfelder for typing the manuscript.

REFERENCES

Davis B. J. (1964) Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121,** 404.

Furlong C. E., Cirakoglu C., Willis R. C. & Santy P. A. (1973) A simple preparative polyacrylamide disc gel electrophoresis apparatus: purification of three branched-chain amino acid binding proteins from *Escherichia coli*. *Analyt. Biochem.* **15,** 297.

Gately M. K., Mayer M. M. & Henney C. S. (1976) Effect of antilymphotoxin on cell-mediated cytotoxicity: evidence for two pathways, one involving lymphotoxin and the other requiring intimate contact between the plasma membrane of killer and target cells. *Cell. Immun.* **27,** 82.

Granger G. A., Laserna E. C., Kolb W. P. & Chapman F. (1973). Human lymphotoxin: purification and some properties. *Proc. natn. Acad. Sci. U.S.A.* **70,** 27.

Granger G. A. & Williams T. W. (1968) Lymphocyte cytotoxicity *in vitro*: activation and release of a cytotoxic factor. *Nature, Lond.* **218,** 1253.

Granger G. A., Yamamoto R. S., Fair D. S. & Hiserodt J. C. (1978). The human LT system: I. Physical–chemical heterogeneity of LT molecules released by mitogen activated human lymphocytes *in vitro*. *Cell. Immun.* **38,** 388.

Hiserodt J. C. & Granger G. A. (1977) Inhibition of human lymphocyte mediated mitogen induced cytotoxicity of murine L-929 cells by heterologous anti-human lymphotoxin antisera *in vitro*. *J. Immun.* **119,** 374.

Hiserodt J. C., Tiangco G. J. & Granger G. A. (1978*a*) The LT system in experimental animals: IV. Rapid specific lysis of allogeneic target cells mediated by highly unstable high molecular weight lymphotoxin receptor complexes released by alloimmune murine T lymphoctyes *in vitro*. *J. Immun.* **123,** 332.

Hiserodt J. C., Yamamoto R. S. & Granger G. A. (1978*b*) The human LT system III: characterization of a large molecular weight LT class (Complex) composed of the various smaller LT classes and subclasses in association with Ig-like molecules. *Cell. Immun.* **38,** 417.

Jeffes E. W. B. III & Granger G. A. (1975) Comparison of proliferation inhibitory factor, cloning inhibitory factor and lymphotoxin. I: growth inhibition and cytotoxicity present in supernatants of mitogen stimulated human lymphocytes. *J. Immun.* **114,** 64.

Klostergaard J. & Granger G. A. (1981) Human lymphotoxins: purification to electrophoretic homogeneity of the α_H receptor-bearing class. *Molec. Immun.* **18,** 455.

Klostergaard J., Yamamoto R. S. & Granger G. A. (1980) Human and murine lymphotoxins as a multicomponent system: progress in purification of the human α_L component. *Molec. Immun.* **17,** 613.

Lee S. C. & Lucas Z. J. (1976) Regulatory factors produced by lymphocytes I. The occurrence of multiple α-lymphotoxins associated with ribonuclease activity. *J. Immun.* **117,** 283.

Lewis J. E., Yamamoto R. S., Carmack C., Lundak R. L. & Granger G. A. (1976) Antibodies against human lymphokines: I. Method for induction of antibodies capable of neutralizing stable (α) and unstable (β) lymphotoxin released *in vitro* by activated human lymphocytes. *J. Immun. Meth.* **14,** 163.

Ross M. W., Tiangco G. J., Horn P., Hiserodt J. C. & Granger G. A. (1978) The LT system in experimental animals: III. Physical–chemical characteristics and relationships of lymphotoxin (LT) molecules released *in vitro* by activated lymphoid cells from several animal species. *J. Immun.* **123,** 325.

Ruddle N. H. & Waksman B. H. (1968) Cytoxicity mediated by soluble antigen and lymphocytes in delayed hypersensitivity III. Analysis of mechanism. *J. exp. Med.* **128,** 1267.

Russel S. W., Rosenau W., Goldberg M. L. & Kunitomi G. (1972) Purification of human lymphotoxin. *J. Immun.* **109,** 784.

Sawada J.-I. & Osawa T. (1978) Cellular cytotoxicity induced by various lectins and mitogens. *Transplant.* **26,** 319.

Tsoukas C. D., Rosenau W. & Baxter J. D. (1976) Cellular receptors for lymphotoxin: correlation of binding and cytotoxicity in sensitive and resistant target cells. *J. Immun.* **116,** 184.

Walker S. M., Lee S. C. & Lucas Z. J. (1976) Cytotoxic activity of lymphocytes. VI. Heterogeneity of cytotoxins in supernatants of mitogen-activated lymphocytes. *J. Immun.* **116,** 807.

Walker S. M. & Lucas Z. J. (1973) Role of soluble cytotoxins

in cell-mediated immunity. *Transplant Proc.* **5,** 137.

Ware C. F. & Granger G. A. (1981) Mechanism of lymphocyte mediated cytotoxicity: I. The effects of anti-human lymphotoxin antisera on the cytolysis of allogeneic B-cell lines by MLC-sensitized human lymphocytes *in vitro*. *J. Immun.* **126,** 1919.

Yamamoto R. S., Hiserodt J. C. & Granger G. A. (1979) The human LT system: V. A comparison of the relative lytic effectiveness of various MW human LT classes on ^{51}Cr-labeled allogeneic target cells *in vitro*. Enhanced lysis by LT complexes associated with Ig-like receptor(s). *Cell. Immun.* **45,** 261.

Yamamoto R. S., Hiserodt J. C., Lewis J. E., Carmack C. E. & Granger G. A. (1978). The human LT system: II. Immunological relationships of LT molecules released by mitogen activated human lymphocytes *in vitro*. *Cell Immun.* **38,** 403.