Title
Human and murine lymphotoxins as a multicomponent system: progress in purification of the human alpha L component.

Permalink
https://escholarship.org/uc/item/3dq890dq

Journal
Molecular immunology, 17(5)

ISSN
0161-5890

Authors
Klostergaard, J
Yamamoto, RS
Granger, GA

Publication Date
1980-05-01

DOI
10.1016/0161-5890(80)90159-5

License
https://creativecommons.org/licenses/by/4.0/ 4.0

Peer reviewed
HUMAN AND MURINE LYMexaoXOINS AS A MULTICOMPONENT SYSTEM: PROGRESS IN PURIFICATION OF THE HUMAN $\alpha_L$ COMPONENT

JIM KLOSTEGAARD, ROBERT S. YAMAMOTO and GALE A. GRANGER
Department of Molecular Biology and Biochemistry, University of California at Irvine, Irvine, CA 92717, U.S.A.

(Received 22 October 1979)

Abstract—Lymphotoxins derived from activated lymphocytes from human and murine lymphoid cells are heterogeneous with respect to molecular size and charge, as well as with respect to the expression of carbohydrate residues. These molecules form a system of interrelated subunits, as evidenced by their shared antigenic determinants, as well as by the reversible dissociation of the smaller forms from the larger. Although the smaller molecular weight forms ($\alpha$, $\beta$, $\gamma$) are apparently only capable of relatively protracted lysis of selected strains of the murine L-929 cell, the higher molecular weight forms ($Cx$, $\alpha_L$) appear to be capable of rapid lysis of the L cell, as well as of relatively rapid, nonspecific lysis of other cells. Furthermore, the Cx forms appear to be associated with an antigen binding receptor which may be of T cell origin. Moreover, these forms released by alloimmune murine T cells can specifically lyse allogeneic tumor cells used in sensitization. The human Cx and $\alpha_L$ LT also appear to express determinants encoded by genes of the MHC. Presently, we have been able to incorporate $^{125}$I into both human and murine lymphotoxin preparations, while fully preserving biological activity. This has enabled us to monitor our attempts at purification of these materials through several consecutive isolation procedures including molecular sieving, ion-exchange chromatography, lectin affinity chromatography, hydrophobic chromatography and electrophoresis. Our results indicate that these materials are present in lymphocyte supernatants in extremely small amounts, probably less than 25 ng/ml; thus the purification of each component by biochemical techniques will require very vigorous methods.

INTRODUCTION

Among the mediators elaborated by activated lymphocytes (lymphokines) is a group of cytotoxins known as lymphotoxin. Previous investigations have shown a wide discrepancy in reported molecular weights and other physical-chemical characteristics of lymphotoxin in every species examined; this has possibly been resolved (Ross et al., 1979). Lymphotoxins have previously only been shown by most laboratories to be capable of protracted nonspecific lysis of the murine L-929 cell(s), causing many investigators not to favor them as candidates for a lytic mechanism mediated by killer T cells against specific targets. Any studies to demonstrate conclusively the presence, much less the critical functioning of these cytotoxins, on the surface of activated killer T cells, necessarily have awaited further biochemical resolution of the soluble forms of these molecules. We review here some recent results from our laboratory aimed at these problems, and also present new strategies and reports of progress toward the eventual resolution of one of the human lymphotoxins.

MATERIALS AND METHODS

Many of the materials and methods employed in these studies have been previously presented in great detail. These will be reiterated briefly here.

Target cells and culture media

The target cells used in these experiments were L-929 (mouse fibroblast). They were maintained as monolayer culture in 32 oz prescription bottles in an atmosphere of 95% air: 5% CO$_2$ and passed biweekly. Culture medium used to maintain L cells was RPMI-1640 (Grand Island Biological Co. (GIBCO), Grand Island, NY, U.S.A.), supplemented with 3% heat-inactivated (1 hr, 56°C) fetal calf serum (FCS, GIBCO), 100 U/ml penicillin and 100 $\mu$g/ml streptomycin (RPMI-S). Suspension cultures of three human lymphoblastoid cell lines, RPMI-1788, WIL-2 and Molt-4, were maintained in RPMI-1640, supplemented with 10% FCS (RPMI 1640 10%).

Lymphocyte cultures and supernatants

(A) Human lymphocytes employed in these studies were obtained from tonsils, adenoids or peripheral blood from normal or immune
donors, as described previously (Yamamoto et al., 1979). Supernatants containing cell lytic activity were also prepared as described previously (Lewis et al., 1977; Yamamoto et al., 1979).

(B) Supernatants obtained from spleen cells of alloimmunized mice were obtained as previously described (Hiserodt et al., 1979).

Physical-chemical separation of LT

Certain methods employed and columns used for fractionation of LT activity have been previously published (Granger et al., 1978). All separation procedures (except where indicated) were carried out at 4°C as rapidly as possible.

Molecular sieving. Briefly, 2 ml of 50 x concentrates of the various supernatants were applied to an Ultrogel AcA 44 column equilibrated in 10 mM potassium phosphate, pH 7.2, and 10^-4 M EDTA. Six milliliter fractions were collected by a Gilson fraction collector at a flow rate of 24 ml/hr.

DEAE-cellulose chromatography. Briefly, rechromatographed LT fractions from several molecular sieving columns were pooled and concentrated. These fractions were dialyzed against starting buffer and applied to a DEAE column equilibrated in 10 mM Tris, pH 8.0, 0.1 mM EDTA. The LT was eluted with a linear 20 ml gradient from 0 to 0.3 NaCl in 10 mM Tris, pH 8.0, 0.1 mM EDTA followed by 1.0 M NaCl in the same buffer. Twenty to thirty drop fractions were collected at a 5-10 ml/hr flow rate and tested for conductivity, and 0.1-0.05 ml tested for LT activity.

Lectin affinity chromatography. Con A-Sepharose was a gift of Dr. Reuben Lotan of the Department of Developmental and Cell Biology, University of California, Irvine, CA, U.S.A. Con A was linked to polyacrylic hydrazide Sepharose (Miles-Yeda, Ltd.) with gluteraldehyde. Radiolabeled or non-radiolabeled human z2 (50-200 µl, 10-80 units), in either Tris buffer or phosphate buffered saline, was allowed to bind Con A-Sepharose (bed volume—250 µ). After 30 min, elution proceeded, first with PBS, and then with 200 mM solutions in PBS of either galactose (for nonspecific desorption) or methyl glucopyranoside (specific elution). The fractions (20-30 drop) were then assayed for radioactivity and lytic activity.

Hydrophobic chromatography. A homologous series of alkyl-agaroses (Shaltiel Hydrophobic Chromatography Kit I, Miles-Yeda, Ltd., Rehovot, Israel) was used to screen for binding to human z2. Fifty to five hundred microliters of z2 (10-200 units) were applied to the series of six columns: agarose, ethyl-agarose, butyl-agarose, hexyl-agarose, octyl-agarose and decyl-agarose. After 30 min, the columns were washed with PBS, collecting 20-30 drop fractions, and the eluates tested for lytic activity on L-929 cells in the standard assay.

Discontinuous polyacrylamide gel electrophoresis (PAGE). PAGE was performed by the method of Davis (1964). A 100-200 µl sample in 20% sucrose was applied to a 0.5 x 8.0 cm gel column consisting of 1 cm 3% acrylamide stacking gel and a 7 cm 7% acrylamide separating gel in 50 mM Tris-glycine, pH 9.6. The sample was subjected to electrophoresis at 4 mA/gel at 4°C. The gels were cut into 2 mm slices, and each slice was incubated in 100-300 µl PBS for 24 hr at 4°C. A sample was then added to 1 ml L cell cultures and tested for LT activity. R values were calculated with reference to the migration of the bromphenol blue marker.

Antisera employed

Rabbit antisera. The details of these procedures are described elsewhere (Yamamoto et al., 1978). Sera employed in these studies were obtained from animals immunized with one of the following preparations: (a) unfractioned serum-free, whole supernatants (anti-WS); (b) a single mol. wt class of LT (anti-α, β, etc.) that has been refined by molecular sieving twice: (c) a single highly-refined LT subclass (anti-α₁, α₂, etc.) that had been refined by molecular sieving twice, DEAE-cellulose chromatography once, and then subjected to PAGE; (d) F(ab')₂ fragments that were prepared from human IgG molecules by pepsin digestion according to methods previously described by Williams & Chase (1967).

Human alloantisera. Anti-HLA-A or B antisera were from multiparous females and were provided by Dr. Roy Walford, Department of Pathology, University of California, Los Angeles, CA, U.S.A. These heat inactivated (60 min, 56°C) sera were dialyzed against PBS and centrifuged to clarity. Various amounts of these or control sera were incubated with 2-5 units of LT activity for 30 min at room temperature. Duplicate samples were then applied to L cells, and the percent neutralization was established as with the rabbit antisera.

Lymphotoxin assay

Two types of assay were employed. One
determined quantitatively the amount of LT activity present in a given supernatant, and one indicated qualitatively its presence or absence. The details of these methods have been reported previously (Spofford et al., 1974). Units of LT activity per milliliter of a given supernatant are obtained by determining the reciprocal of the dilution killing 50% of the target L cells.

**Antibody neutralization tests**

Each serum was first tested to determine its effectiveness at neutralizing a known amount of LT activity on L cells in vitro. Doses of antisera to neutralize a given amount of LT have been previously described by Yamamoto et al., 1978.

**Protein iodination**

*Iodogen.* The procedure of Fraker & Speck (1978) was essentially followed in these experiments. 1,3,4,6-tetrachloro-3,6-diphenylglycoluril (Pierce) (0.1–10 μg) in 10–100 μl spectrophotometric grade methylene chloride (Mallinckrodt, St. Louis, MO, U.S.A.) was added to 10 x 75 mm test tubes, and the solvent evaporated with a nitrogen stream. Lymphotoxin preparation (50 μl–2 ml) were added to the dried tubes, followed rapidly by 100–500 μCi 125I in phosphate buffered saline. The reaction was terminated by decanting after 90 sec to 10 min, and then the preparation was dialyzed against PBS with 10–3 M KI. Efficiencies varied from ~10 to 70%.

**Fluorescamine assay.** Protein samples (0.25–1.0 ml) in phosphate buffer (pH range 7-8.5, 0.01–0.1 M) were rapidly mixed with 0.5 ml fluorescamine (Aldrich) (30 mg in 100 ml dry dioxane). Bovine serum albumin Fraction V (Miles, Kankakee, IL, U.S.A.) was used as a standard. Fluorescene determinations were made on a Perkin–Elmer Model MPF-3L Fluorescence Spectrophotometer.

**RESULTS**

**The multicomponent nature of lymphotoxin**

Identification of human and murine molecular weight LT classes. Concentrated whole supernatants from human adenoid or peripheral blood lymphocytes activated by lectin or alternatively primed in MLC culture prior to lectin stimulation were chromatographed on Ultrogel AcA 44. A typical elution profile is shown in Fig. 1A. Nonadherent spleen cells from C57B1/6 mice were activated by a PHA-coated L cell monolayer. The concentrated supernatants were chromatographed in the same manner as the human material. This profile is shown in Fig. 1B. It is clear that the human cytotoxic activity can be resolved into several discrete molecular weight classes: Cx, >200,000 daltons (d); CI~, 70,000–90,000 d; CI~3, 35,000–50,000 d; and γ, ~15,000 d. The Cx and CI~ toxic activities appear to be very stable under conditions of storage at low salt and 4°C. Murine cytotoxic activity also appears in several forms of distinct molecular weights. Aside from the difference in proportion of activities found, the predominant activity is the Cx class (150,000 daltons) in early release murine supernatants (Hiserodt et al., 1979); however, α and β predominate in late supernatants. These activities are highly unstable, rapidly losing potency even by storage at 4°C.

Identification of human and murine LT charge subclasses. Pooled concentrates of human α class lytic activity were chromatographed on a DEAE-cellulose column. The lytic activity profile is seen in Fig. 2A. The first subclass, α1, appears in the breakthrough fractions, while the second and most significant subclass, α2, is desorbed from the column at a NaCl concentration of about 0.05 M. The α3 activity is eluted in the 1 M NaCl wash. Human β class activity may be similarly resolved into two subclasses: β1, in the breakthrough fraction, an unstable activity; and β2, eluted on the salt gradient, a stable
activity. The murine \( \alpha \text{H}_{1} \) activity can be similarly resolved in three subclasses (Fig. 2B). Two subclasses, \( \alpha \text{H}_{2a} \) and \( \alpha \text{H}_{2b} \), are eluted from DEAE-cellulose with a salt gradient, and \( \alpha \text{H}_{3} \) with 1 M NaCl.

**Antigenic relationships between components of the lymphotoxin system.** Heterologous rabbit antisera to human lymphotoxins were tested for their ability to neutralize the lytic capacity against L-929 cells of various classes and subclasses of lymphotoxins. These results are summarized in Table 1. This is clearly a very complex pattern of immunological reactivities. It should simply be pointed out that each class and subclass may carry both public and private antigenic specificities. Studies conducted in the guinea pig and mouse reveal that a similar pattern of immunological reactivities exist (Hiserodt et al., 1979; Ross et al., 1979). This finding of public specificities expressed by classes and subclasses of lymphotoxins was the first evidence that they comprised a system of related subunits.

**Evidence for the association of antigen-binding receptors with human and murine lymphotoxin activities.** Heterologous anti-F(ab')\(_2\) antisera were tested for ability to neutralize the lytic activity against L-929 cells expressed by several human LT classes. Various goat anti-human heavy chain specific antisera were also tested for their blocking ability on human LT complex. These results are summarized in Table 2. Only the Cx class from lectin stimulated lymphocytes appears to be blocked significantly by anti-F(ab')\(_2\) antisera, and this does not appear to be due to the expression of classical Ig determinants, since the anti-heavy chain antisera are totally without a blocking effect.

**Table 1. Reactivity of anti-LT antisera with LT activities in whole supernatants and in selected fractions**

| Antiserum employed | Whole supernatant | Class \( \alpha \text{L} \) | Subclass \( \beta \) | \( \gamma \) | \( x_1 \) | \( x_2 \) | \( x_3 \) |
|--------------------|------------------|-----------------|-----------------|-----|-----|-----|-----|
| Anti-Cx           | +                | ++              | ++              | ++  | ++  | ++  | ++  |
| Anti-\( \alpha \text{L} \) | +                | ++              | ++              | +   | ++  | +   | +   |
| Anti-\( \alpha \text{L} \) | ++              | ++              | ++              | +   | ++  | +   | +   |
| Anti-\( \alpha \text{L} \) | ++              | ++              | ++              | +   | ++  | +   | +   |
| Anti-\( \alpha \text{L} \) | ++              | ++              | ++              | +   | ++  | +   | +   |

*Neutralization refers to inactivation of 200–300 units of LT activity by 100 \( \mu \)l antisera: \(- = 0–15\%\) \( + = 15–40\%\) \( + + = 40–80\%\) \( + + + = 80–100\%\) neutralization.

*NT = Not tested.*

**Table 2. Neutralizing effect of various heterologous anti-human Ig antisera on human LT classes**

| Antiserum employed | Per cent neutralization of LT classes |
|--------------------|-------------------------------------|
|                    | \( \alpha \text{L} \) | \( \alpha \) | \( \beta \) |
| Rabbit anti-F(ab')\(_2\) | 58 ± 6 | 5 | 11 ± 2 |
| Rabbit anti-F(ab')\(_2\) IgG fraction | 68 ± 4 | NT* | NT |
| Goat anti-\( \gamma \) chain | 0 | NT | NT |
| Goat anti-\( \delta \) chain | 0 | NT | NT |
| Goat anti-\( \gamma \), \( \mu \), \( \delta \) chain | 1 | NT | NT |

*NT = Not tested.*
Table 3. Induction of antigen-specific LT activity in lymphocyte whole supernatants by soluble or cellular antigens

| Human immune lymphocyte donor | Agent inducing LT | Per cent of LT activity bound to antigen beads |
|-------------------------------|------------------|-----------------------------------------------|
|                               |                  | TT                                           |
| A                             | SKSD             | 36 ± 9                                       |
|                               | Con A            | 5 ± 5                                        |
| B                             | SKSD             | 39 ± 6                                       |

Table 4. Specific killing of allogeneic targets by murine alloimmune lymphocyte supernatants

| Immune lymphocytes | Method activation | P815 % 51Cr release | Supernatant killing EL4 % 51Cr release | L.T (units/ml) |
|--------------------|------------------|----------------------|----------------------------------------|----------------|
| C57Bl/6 α-P815     | PHA/L cell       | (Unconc.) 19 ± 1     | 4 ± 0.1                                | 86             |
| C3H/DbSn α-P815    | PHA/3T3          | (10 x conc.) 15 ± 1  | 4 ± 1                                  | 780            |
| D2A/2 α-EL4        | PHA/L cell       | (10 x conc.) 2 ± 0.6 | 36 ± 2                                 | 900            |
| C3H/DbSn α-EL4     | PHA/HeLa         | (10 x conc.) 2 ± 0.1 | 18 ± 2                                 | NT             |

We then tested the concept that antigen-stimulated human lymphocytes could elaborate LT activity associated with Ig-like antigen-binding receptors. First, we tested whether LT activity in supernatants from immune peripheral blood lymphocytes stimulated with soluble antigen could be absorbed by immobilized specific antigen. Then we examined whether the activity in supernatants from MLC primed lymphocytes could be absorbed by the stimulator cells. These results are shown in Table 3. It is apparent that either soluble or cellular antigens induce a very significant proportion of LT activity in lymphocyte supernatants which is capable of specifically recognizing the antigen used in induction. This proportion is much greater than when a polyclonal activator is used to stimulate the lymphocytes.

Further evidence for antigen-specific lymphotoxins was obtained in the murine system employing supernatants from alloimmune splenocytes. Supernatants from cultures of C57Bl/6 and C3H/DbSn spleen cells, alloimmunized to the P815 mastocytoma, and supernatants from DBA/2 and C3H/DbSn spleen cells, immune to the EL4 lymphoma, were tested for their lytic activity on the L-929 cell, as well as on the specific target and a nonrelated target. Typical results are shown in Table 4. Due in part to the extreme lability of the cytotoxic activities in murine supernatants, not every experiment conducted gave testable levels of killing. However, in those shown, a strong and specific cytolytic activity against the stimulating allogeneic target could be seen. The α L-929 cell reactivity to receptor and non-receptor forms of...
LT reflects its unique sensitivity to all LT forms.

Evidence that the specific lytic forms are of T cell origin. In order to attempt to ascertain the cellular origin of the antigen-specific killing forms in murine supernatants, various manipulations of the responding splenocyte population were attempted. These results are shown in Table 5. As can be seen, removal of adherent cells from the splenocyte population increases the levels of soluble cytotoxic activity, whereas depletion of \( \theta \)-positive cells results in no activity being detectable. A nylon wool 'purified' T cell preparation, as described by Julius et al. (1973), alone appears to be fully capable of elaborating the activity.

Association of MHC gene products with human LT activities. Lymphotoxin activities from all the human LT classes were tested for their expression of HLA-A or B loci products by neutralization with anti-HLA antisera. These results are presented in Table 6. Both the Cx and \( \gamma_1 \) classes are consistently blocked by anti-HLA antisera reactive with the haplotype expressed by the lymphocyte donor. The \( \gamma_2 \) class is also blocked to some degree by these sera. Surprisingly, an anti-HLA antisera, anti-A1, presumably unreactive with the lymphocyte donor haplotype, also showed significant blocking of Cx, \( \gamma_1 \) and \( \beta \)-LT. This may reflect the association of alloantigens distinct from HLA-A and B with LT components.

Radiolabeling and purification of human lymphotoxin preparations

Establishing conditions for labeling. We have previously experienced extreme difficulty in effectively radiolabeling lymphotoxins. Most of the oxidative methods for the introduction of radioiodine result in concomitant loss of biological effects of the protein, i.e. lytic capacity (unpublished results). Our results with the Bolton–Hunter reagent have been fraught with unforeseen technical problems (Klostergaard & Mayers, in preparation). The use of \( ^3 \)H or \( ^14 \)C-labeled amino acids for internal labeling of biosynthesized products during culture have yielded LT preparations with inadequate specific activities.

We have found the solid phase iodination technique introduced by Fraker & Speck (1978) effective in preserving activity in our LT preparations, while allowing us to label to a sufficient level of radioactivity. Initial labeling experiments were conducted on an \( \alpha_2 \) preparation in order to establish conditions for labeling. These results are summarized in Table 7.

At the highest ratios of protein (\( \mu \)g) to iodogen (\( \mu \)g) investigated (300), lytic capacity was fully preserved at the 5 min exposure time. At lower ratios (50) and longer exposure time (10 min), lytic activity was partially compromised. In recent experiments with this and other LT preparations, we have obtained further evidence that this ratio is quite critical and probably depends on the inherent sensitivity of the macromolecule to oxidizing conditions. It may also depend on the degree of purity of the preparation being labeled, with contaminating proteins perhaps ‘buffering’ the macromolecule

| Lymphocyte donor HLA-type | Antisera employed | Cx | Per cent neutralization of LT class \( \gamma_H \) \( \gamma_L \) \( \beta \) |  
| Exp. 1 A—3; 11 B—15; 35 | Anti-A | 44 ± 7 | 6 ± 2 | 10 ± 3 | 7 ± 3  
| Exp. 2 A—29; 30 B—12; 13 | Anti-B, Anti-B, 3 | 44 ± 6 | 38 ± 2 | NT | 12 ± 2 | 0  
| | Anti-B, Anti-A, 3 | 47 ± 10 | 41 ± 5 | NT | 20 ± 2 | 0  
| | Anti-A, 3 | 29 ± 6 | 28 ± 3 | NT | 29 ± 5 | 0  

\*NT = Not tested.

Table 7. Effect of iodogen levels and time of exposure on both the efficiency of labeling and on preservation of human \( \alpha_2 \) lytic activity

| Volume of \( \alpha_2 \) (\( \mu \)l) | Iodogen plated (\( \mu \)g) | Duration of labeling (min) | Efficiency of labeling (\%\) | Lytic activity (\%\) |  
| --- | --- | --- | --- | --- |  
| 500 | 3 | 10 | ± 25 | 95  
| 500 | 10 | 10 | ± 30 | 85  
| 150 | 1 | 5 | ± 25 | 100  
| 150 | 0.5 | 5 | ± 25 | 100  

Table 6. Effect of alloantisera on cytotoxic activity expressed in different human LT classes

| Lymphocyte donor HLA-type | Antisera employed | Cx | Per cent neutralization of LT class \( \gamma_H \) \( \gamma_L \) \( \beta \) |  
| Exp. 1 A—3; 11 B—15; 35 | Anti-A | 44 ± 7 | 6 ± 2 | 10 ± 3 | 7 ± 3  
| Exp. 2 A—29; 30 B—12; 13 | Anti-B, Anti-B, 3 | 44 ± 6 | 38 ± 2 | NT | 12 ± 2 | 0  
| | Anti-B, Anti-A, 3 | 47 ± 10 | 41 ± 5 | NT | 20 ± 2 | 0  
| | Anti-A, 3 | 29 ± 6 | 28 ± 3 | NT | 29 ± 5 | 0  

Radiolabeling and purification of human lymphotoxin preparations

Establishing conditions for labeling. We have...
of interest against unfavorable reaction conditions. We fully realize that our LT preparations are still highly impure, and presumably only a small proportion of the label in a preparation has actually been introduced into the lymphotoxin molecule.

**Labeling and molecular sieving of whole supernatant.** In order to verify our assumption that in our isolation and purification experiments we could use radioactivity as a very sensitive assay for protein, we conducted the following initial experiment. A 50-fold concentrated whole supernatant from a 5-day culture of PHA-stimulated adenoid lymphocytes was dialyzed against PBS overnight. A 15 ml sample was iodinated, using 1 μg of Iodogen, and ~500 μCi 125I. After overnight dialysis against starting buffer, the labeled whole supernatant was fractionated on Ultrogel Aca 44, as described in Materials and Methods. The fractions were then assayed for radioactivity (in 100 μl), 50 μl were tested for toxicity on L cells, and 1 ml was assayed for protein concentration against a BSA standard, using the fluorescamine assay. The results are shown in Fig. 3.

It is apparent that although the fluorescamine assay and the radioassay depend on entirely different characteristics of the proteins present in the whole supernatant, the assays are in excellent agreement throughout the molecular weight range studied. By far the greatest amount of the protein is in the void volume, presumably reflecting the preponderance of proteins from the serum substitute added to the lymphocyte culture (Lewis et al., 1977). We have also found that PHA is a significant contaminant in the range from near the void to ~10,000 daltons (data not shown).

**Labeling and purification of a class LT on DEAE-cellulose.** To ascertain the purification of x1 achieved by chromatography on DEAE-cellulose, 200 μl of x1 was iodinated with 1 μg of Iodogen and ~500 μCi 125I with a 5 min

| Source                          | LT activity (units) | Protein | Specific activity (units/μg protein) |
|---------------------------------|--------------------|---------|-------------------------------------|
| 1.5 ml Whole supernatant x (50 x concentrate) | ≈1000              | ≈300    | ≈3                                 |
| 200 μl x1 (10 x concentrate) on DEAE-cellulose | ≈125               | ≈5 μg   | ≈25                                |
| 50 μl x1 on Con A-Sepharose      | ≈25                | ≈200 ng | ≈125                               |
| 50 μl x1 on PAGE after Con A-Sepharose | ≈20                | ≈20 ng  | ≈1000                              |
reaction. After multiple dialysis changes against starting column buffer, the labeled $x_1$ was loaded onto the column, and the column was developed as described in Materials and Methods. Each fraction was then assayed for lytic activity (100 $\mu$l) and radioactivity (100 $\mu$l). In Fig. 4, the peak of major lytic activity, $x_2$, is desorbed on the same gradient which removes most of the labeled protein. However, the gradient elution clearly allows us to judiciously pool only those fractions displaying great lytic activity and relatively little protein, in this case, fractions 18–20. PAGE reveals that despite this level of purification over what is found in the whole supernatant (Table 8), the $x_2$ preparation is overwhelmingly dominated by contaminating proteins (data not shown).

**Purification of labeled $x_2$ on lectin columns.** Previous studies from this laboratory (Toth & Granger, 1979) have shown that human $x_2$ is a glycoprotein; it is heterogeneous with respect to expression of carbohydrate, with 50% or more of the lytic activity being bound by Con A-Sepharose. We have exploited this property of $x_2$ and used Con A-Sepharose columns to purify the glycoprotein in the following manner: 50 $\mu$l of a 500 $\mu$l preparation of $x_2$, which had been iodinated with 3 $\mu$g of Iodogen with a 10 min reaction time, was applied to the Con A-Sepharose column as described in Materials and Methods. After allowing adsorption, the column was washed sequentially with PBS, 200 mM galactose in PBS, 200 mM methyl-glucopyranoside in PBS, and then 200 mM methyl-glucopyranoside in 0.5 M NaCl in PBS. The fractions were assayed for radioactivity (10 $\mu$l) and lytic activity (200 $\mu$l). The results are shown in Fig. 5.

Most of the protein and some of the lytic activity (~10%) appears in the breakthrough fractions. A very small amount of lytic activity and protein is desorbed with the nonspecific sugar, galactose, perhaps reflecting disruption of hydrogen bonding interactions between applied protein and the column material. A majority (>60%) of the lytic activity is eluted with the specific sugar, methyl-glucopyranoside, while only simultaneously desorbing ~20% of the total protein applied. Further lytic activity (~30%) and protein (<10%) is eluted in the presence of the specific sugar and 0.5 M NaCl. This type of nonspecific interaction between glycoproteins and lectin columns has previously been shown by other investigators (Davey et al., 1974).

**PAGE of labeled lectin purified $x_2$.** In order to determine if the lectin affinity separation of $x_2$ resulted in a homogeneous product, we conducted the following experiment. Two hundred microliters of $x_2$ was further purified on a Con A-Sepharose column as previously detailed. The first fraction collected in the methyl-glucopyranoside wash was labeled with 1 $\mu$g of Iodogen for 5 min with 500 $\mu$Ci $^{125}$I. After dialysis against 500 vol. PBS for 1 hr, 300 $\mu$l of the iodinated protein was resolved by PAGE as described in Materials and Methods. The gel slices were eluted with PBS + 1% lactalbumin hydrolysate overnight at 4°C. The radioactivity in each slice was determined, and then 200 $\mu$l of
the eluate was assayed on L cells. The result is shown in Fig. 6.

The lytic activity peak appears to correspond to a very minor peak of radioactivity at Rf ~ 0.4. Obviously, this is still a very impure preparation, which is dominated by a large protein peak at Rf ~ 0.7.

Hydrophobic chromatography of \( \alpha_2 \). We have begun to explore the utility of hydrophobic chromatography in purifying lymphotoxins. Early work with the human \( \alpha_2 \) consisted of a screening procedure employing a series of alkyl-Sepharose columns of varying chain lengths (see Materials and Methods). On each of the \( C_n-C_{1n} \) alkyl columns was loaded 100 \( \mu l \) of \( \alpha_2 \). After binding had occurred, the columns were washed with PBS, and the eluted fractions were assayed for LT activity. As seen in Fig. 7, columns substituted with alkyl chains as long as n-hexyl were unable to bind the LT activity. In contrast, octyl-Sepharose retarded the activity, and decyl-Sepharose bound it. We are currently in the process of determining and optimizing protocols for elution of the lytic activity from the decyl-Sepharose column.

**DISCUSSION**

It is clear that lymphotoxins must be viewed in a new perspective. Previous studies from this laboratory have strongly documented the fact that lymphotoxins (LT) from the human and a variety of animal species constitute a system of related subunits which appear to have been largely preserved through evolution. Within a species, the subunits display a complex pattern of both shared and distinct antigenic determinants. The molecular basis for this crossreactivity may be attributed in part to the fact that the various components appear to form lytically active multimers; thus, the \( \alpha_2 \) and \( \alpha_4 \) LT forms appear to be comprised in part of the \( \alpha_4 \). \( \beta \) and \( \gamma \) components, which may be dissociated through perturbation of weak, noncovalent bonds. An antigen-specific \( \alpha_2 \) form has been demonstrated in both the human and murine systems. Recent evidence (Harris P. & Granger G. A., manuscript in preparation) has shown that the human \( \alpha_4 \) form (150,000 d) also appears to have receptor activity. It is noteworthy that other investigators have also reported on molecules from T cells with receptor activity which are in the same molecular weight range (Binz & Wigzell, 1977; Krawinkel et al., 1977). It appears that the enhanced killing found for \( \alpha_2 \) and \( \alpha_4 \) forms may arise as a result of the focusing of the lytic capacity of individual subunits; in contrast, the smaller molecular weight forms are only weakly lytic. Furthermore, both the human \( \alpha_2 \) and \( \alpha_4 \) form express determinants encoded by the MHC.

While these studies were still in their infancy, it became very apparent that LT(s), as is probably the case with all of the lymphokines, were present in extremely small amounts. Thus, two major approaches to the biochemical studies and purification of these mediators were tenable. Either one must routinely generate enormous quantities of lymphocyte supernatants for further study, or alternatively, viable micro-methods for isolation and purification had to be developed. Only by simultaneously being able to monitor biological activity, as well as protein, could a determination be made as to the efficacy of a particular isolation procedure.

For the last several years, we have expended considerable effort in adopting suitable radiochemical tagging procedures to serve as a protein monitor. Whereas internal labeling methods employing \(^3\)H- or \(^14\)C-amino acids have a great advantage in only being incorporated by proteins synthesized during incubation, the low specific activity (CPM) of the isotopes, and caveats of employing them in culture, as well as of liquid scintillation counting, have limited their usefulness in our hands. The use of \(^125\)I in

---

**Fig. 7.** Effect of alkyl chain length on adsorption of human \( \alpha_2 \) to hydrophobic columns. Human \( \alpha_2 \) was applied to each of a series of alkyl columns: \( C_0 \)-unsubstituted Sepharose (○), \( C_2 \)-ethyl Sepharose (♦), \( C_6 \)-n-butyl-Sepharose (■), \( C_7 \)-n-hexyl-Sepharose (□), \( C_8 \)-n-octyl-Sepharose (△) and \( C_{10} \)-n-decyl Sepharose (▲). After allowing binding, the columns were developed with PBS, and the lytic activity in each fraction determined as indicated.
external tagging of proteins increases the complexity of the labeled preparation, since both synthesized proteins and exogeneous proteins may be labeled. However, the short half-life of the radioisotope facilitates obtaining preparations of high specific activity. While we have therefore pursued the latter path more vigorously, an insurmountable obstacle until recently was the fact that any of the methods we chose for introduction of radiiodine to LT preparations had lethal effects on the biological activity of the molecule(s).

In our hands, neither the chloramine-T method nor the lactoperoxidase method allowed a retention of LT lytic activity following labeling (unpublished results). It was our feeling that since the LT molecule(s) was obviously denatured to some extent by these labeling procedures, it would be folly to combine labeled and unlabeled preparations with the expectation that the labeled but denatured LT molecule would behave identically to the unlabeled but active molecule in all isolation procedures needed for complete resolution. We thus turned to the milder method for radioiodinating our preparations.

The use of the Bolton–Hunter reagent in labeling of polypeptide hormones (Bolton & Hunter, 1973) and other proteins has given it wide acceptance as a method of choice for tagging of proteins. We initially utilized this reagent in our attempts to radiolabel LT preparations. Although we were encouraged by the fact that biological activity was preserved, the labeling efficiency was very low. We have subsequently abandoned this technique, as we have found it unsuitable for introducing a stable radiolabel in a number of proteins (Klostergaard J. & Mayers G. L., manuscript in preparation). The Iodogen method, introduced by Fraker & Speck (1978), has allowed us to introduce a stable radiolabel into several LT preparations, with a suitable efficiency, and with complete preservation of lytic activity. Based on our experience with a labile biological activity and the potentially harmful oxidative conditions encountered while labeling, we caution other investigators to be exacting in establishing those conditions (protein nature, concentration and volume; Iodogen level, time of exposure, etc.) under which they may achieve suitable labeling efficiency while preserving biological activity.

We must stress the significance of our successful application of the Iodogen labeling method as a powerful micro-technique in our goal to purify to homogeneity the components of the human LT system. As seen in Table 8 and Fig. 6, even after three consecutive purification procedures, i.e. molecular sieving, ion-exchange chromatography and lectin affinity chromatography, human $\alpha_2$ is still extremely inhomogenous, while the specific activity has risen several hundred-fold over the activity found in the whole supernatant. It is our ability to monitor simultaneously protein concentration and lytic activity which has allowed us to determine that our purification procedures have, in fact, resulted in LT preparations of much higher specific activity. At the same time, it has clearly shown us that we need even more innovative methods for purifying LT(s). For example, we have an enormous contaminating component in the $\alpha_2$ preparation, purified on Con A-Sepharose, as seen in PAGE ($R_f \approx 0.7$). As indicated in Fig. 7, we are actively pursuing other chromatographic procedures for the further purification of these preparations, with some success. Among the strategies being employed in our laboratory is the potential use of monoclonal antibodies reactive with the particular LT components for purification of the molecules. We are very excited about the prospects of these new purification procedures in conjunction with radiolabeling. This should allow us to answer a great variety of critical questions, ranging from identifying an antigen-specific receptor of probable T cell origin, to examining the interaction between the killer lymphocyte and the target cell with LT-specific reagents.

Note added in proof—Since the preparation of this manuscript, we have isolated from a radioiodinated $\alpha_2$ preparation, a labeled protein which comigrates with $\alpha_2$ lytic activity in electrophoresis. This protein appears homogeneous in two sequential electrophoretic procedures. Experiments to verify further the identity of this protein with $\alpha_2$ lymphotoxin molecules are under way in our laboratory.

REFERENCES

Binz H. & Wigzel H. (1977) Antigen-binding, idiotypic T-lymphocyte receptors. Contemp. Topics Immunobiol. 7, 113.

Bolton A. E. & Hunter W. M. (1973) The labelling of proteins to high specific radioactivities by conjugation to a $^{125}I$-containing acylating agent—Application to radioimmunoassay. Biochem. J. 133, 529.

Davey M. W., Huang J. W., Sulkowski E. & Carter W. A. (1974) Hydrophobic interaction of human interferon with concanavalin A-agarose. J. biol. Chem. 249, 6354.
Davis B. J. (1964) Disc electrophoresis—II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. 121, 404.

Fraker P. J. & Speck J. C. (1978) Protein and cell membrane iodinations with a sparingly soluble chloramide, 1,3,4,6-tetrachloro-3a,6a-diphenyl-glycoluril. Biochem. biophys. Res. Commun. 80, 640.

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., Tiaanco G. J. & Granger G. A. (1979a) The LT system in experimental animals: II. Physical and immunological characteristics of molecules with LT activity rapidly released by murine lymphoid cells activated on lectin-coated allogeneic monolayers in vitro. J. Immun. 123, 317.

Hiserodt J. C., Tiaanco G. J. & Granger G. A. (1979b) 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 allogeneic murine T lymphocytes in vitro. J. Immun. 123, 332.

Julius M. H., Simpson E. & Herzenberg L. A. (1973) A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur. J. Immun. 3, 645.

Krawinkel U., Cramer M., Imanishi-Kari T., Jack R. S. & Rajewsky K. (1977) Isolated hapten-binding receptors of sensitized lymphocytes I. Receptors from nylon wool-enriched mouse T lymphocytes lack serological markers of immunoglobulin constant domains but express heavy chain variable portions. Eur. J. Immun. 7, 566.

Lewis J. E., Carmack C. E., Yamamoto R. S. & Granger G. A. (1977) Antibodies against human lymphokines: I. Method for induction of antibodies capable of neutralizing stable (a) and unstable (b) lymphotoxins released in vitro by activated human lymphocytes. J. Immunol. Meth. 14, 163.

Ross M. L., Tianango G. J., Horn P., Hiserodt J. C. & Granger G. A. (1979) 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 animals species. J. Immun. 123, 325.

Spofford B., Daynes R. A. & Granger G. A. (1974) Cell-mediated immunity in vitro. A highly sensitive assay for human lymphotoxin. J. Immun. 112, 2111.

Toth M. K. & Granger G. A. (1979) The human LT system: VI. Identification of various saccharides on LT molecules and their contribution to cytotoxicity and charge heterogeneity. Molec. Immun. 16, 67.

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.

Williams C. A. & Chase M. W. (1968) Methods in Immunology and Immunochemistry, Vol. I. Academic Press, New York.

Williams T. W. & Granger G. A. (1973) Lymphocytes in vitro cytotoxicity: mechanisms of human lymphotoxin-induced target cell destruction. Cell. Immun. 6, 171.

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.

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 %Cr-labeled allogeneic target cells in vitro. Enhanced lysis by LT complexes associated with Ig-like receptor(s). Cell. Immun. 45, 261.