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HUMAN AND MURINE LYMPHOTOXINS AS A MULTICOMPONENT SYSTEM: PROGRESS IN PURIFICATION OF THE HUMAN α_L COMPONENT

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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 (α_L, β, γ) are apparently only capable of relatively protracted lysis of selected strains of the murine L-929 cell, the higher molecular weight forms (Cx, α_H) 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 α_H 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 μg/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× concentrates of the various supernatants were applied to an Ultrogel AcA 44 column equilibrated in 10 m*M* 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 m*M* Tris, pH 8.0, 0.1 m*M* EDTA. The LT was eluted with a linear 20 ml gradient from 0 to 0.3 NaCl, in 10 m*M* Tris, pH 8.0, 0.1 m*M* 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 α_2 (50–200 μl, 10–80 units), in either Tris buffer or phosphate buffered saline, was allowed to bind Con A-Sepharose (bed volume—250 μl). 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 α_2. Fifty to five hundred microliters of α_2 (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 0.5×8.0 cm gel column consisting of 1 cm 3% acrylamide stacking gel and a 7 cm 7% acrylamide separating gel in 50 m*M* 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_f 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-α_1, α_2, etc.) that had been refined by molecular sieving twice, DEAE-cellulose chromatography once, and then subjected to PAGE; (4) $F(ab')_2$ 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-3α,6α-diphenyl-glycoluril (Pierce) (0.1–10 μg) in 10–100 μl spectrophotometric grade methylene chloride (Mallinckrodt, St. Louis, MO, U.S.A.) was added to 10 × 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 ^{125}I 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

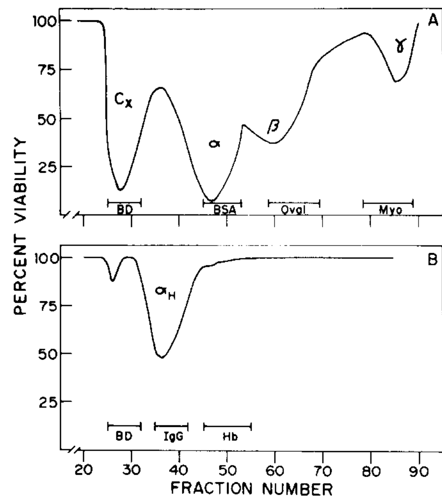

Fig. 1. Molecular sieving of human (A) and murine (B) lymphocyte whole supernatants on Ultrogel AcA 44. The viability of L cells following incubation with an aliquot of every other fraction as described in Materials and Methods is indicated. Markers: Blue Dextran (BD), bovine serum albumin (BSA), ovalbumin (Oval), myoglobin (Myo), immunoglobulin (IgG) and hemoglobin (Hb).

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); $α_L$, 70,000–90,000 d; β, 35,000–50,000 d; and γ, ~15,000 d. The Cx and $α_L$ 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 $α_H$ 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, $α_1$, appears in the breakthrough fractions, while the second and most significant subclass, $α_2$, is desorbed from the column at a NaCl concentration of about 0.05 *M*. The $α_3$ activity is eluted in the 1 *M* NaCl wash. Human β class activity may be similarly resolved into two subclasses: $β_1$, in the breakthrough fraction, an unstable activity; and $β_2$, eluted on the salt gradient, a stable

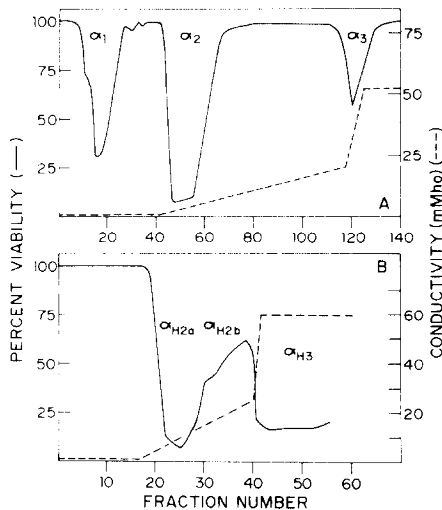

Fig. 2. DEAE-cellulose chromatography of α-class lymphotoxins. Human α_L (A) was chromatographed, using a linear 0–0.3 *M* NaCl gradient in starting 10 m*M*, pH 8 Tris buffer (fractions 40–120), followed by a 1 *M* NaCl wash (121–140). Murine α_H (B) was chromatographed similarly; a linear gradient (0–0.4 *M* NaCl) was applied from fraction 18–40, and the 1 *M* NaCl wash from 41–60. Samples from alternate fractions were assayed on α L-929 cells as described in Materials and Methods. Toxicity in the murine α_{H3} peak is due in part to high amounts of NaCl added to L cell cultures.

activity. The murine α_H activity can be similarly resolved in three subclasses (Fig. 2B). Two subclasses, α_{H2a} and α_{H2b}, are eluted from DEAE-cellulose with a salt gradient, and α_{H3} 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

Human	Human LT preparation									
Antiserum employed	Whole supernatant	Class			Subclass					
		Cx	α_L	β	γ	α_1	α_2	α_3	β_1	β_2
Anti-Cx	+ [a]	+ +	+ + +	+	−	+ +	+	−	NT [b]	NT
Anti-α_1	+	+ +	+	−	−	+ +	−	−	NT	NT
Anti-α_2	+ + +	+ +	+ +	+	+ +	+ + +	+ + +	+ +	NT	NT
Anti-β_1	NT	+ + +	−	+ +	−	−	−	−	+ +	−
Anti-β_2	NT	+ + +	+ +	+ +	+	+ +	+ +	+ +	−	+ +

[a] Neutralization refers to inactivation of 200–300 units of LT activity by 100 μl antisera: − = 0–15%, + = 15–40%, + + = 40–80%, + + + = 80–100% neutralization.
[b] 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 Cx	α	β
Rabbit anti-F(ab′)$_2$	58±6	5	11±2
Rabbit anti-F(ab′)$_2$ IgG fraction	68±4	NT [a]	NT
Goat anti-γ chain	0	NT	NT
Goat anti-δ chain	0	NT	NT
Goat anti-γ, μ, δ chain	1	NT	NT

[a] 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	SKSD	BSA
A	TT	36±9	9±6	5±5
	SKSD[b]	5±5	33±9	2±2
	Con A	5±5	5±5	5±2
B	TT	39±6	3±2	NT
	SKSD	4±2	33±5	3±1

Human lymphocyte donor	Agent inducing LT	Autologous lymphocytes	Per cent of LT activity absorbed by cell lines			
			RPMI-1788	WIL-2	Molt-4	L-929
C	RPMI-1788	3	46	12	8	8
	WIL-2	5	8	62	10	9
	Molt-4	NT[a]	8	NT	39	NT
	SKSD	NT	13	14	NT	NT
	Con A	NT	18	16	NT	10

[a] NT = Not tested.
[b] SKSD = Streptokinase-streptodornase.

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/DiSn spleen cells, alloimmunized to the P815 mastocytoma, and supernatants from DBA/2 and C3H/DiSn 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

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

Immune lymphocytes	Method activation	Supernatant killing: P815 % ^{51}Cr release	Supernatant killing: EL4 % ^{51}Cr release	LT (units/ml)
C57Bl/6 α-P815	PHA/L cell	(Unconc.) 19±1	4±0.1	86
C3H/DiSn α-P815	PHA/3T3	(10× conc.) 15±1	4±1	780
DBA/2 α-EL4	PHA/L cell	(10× conc.) 7±0.6	36±2	900
C3H/DiSn α-EL4	PHA/HeLa	(10× conc.) 2±0.1	18±2	NT

Table 5. Requirement for T cells for soluble specific anti-P815 cell lytic activity released *in vitro* by C57Bl/6 splenocytes alloimmune to P815

Treatment of lymphoid cells	% ^{51}Cr release from P815 targets
Unseparated spleen	18–23
Non-glass adherent lymphocytes	15–30
Non-glass adherent lymphocytes + anti-θ + complement	2–4
Non-glass adherent nylon wool purified lymphocytes	19–29

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

Lymphocyte donor HLA-type		Antisera employed	Per cent neutralization of LT class				
			Cx	α_H	α_L	β	γ
Exp. 1	A—3; 11 B—15; 35	Anti-A_3	44±7	67±5	7±2	10±23	7±3
Exp. 2	A—29; 30	Anti-B_{12}	44±6	38±2	NT[a]	12±2	0
	B—12; 13	Anti-B_{13}	47±10	41±5	NT	20±2	0
		Anti-A_{11}	29±6	28±3	NT	29±5	0

[a] NT = Not tested.

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 θ-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 α_H classes are consistently blocked by anti-HLA antisera reactive with the haplotype expressed by the lymphocyte donor. The β class is also blocked to some degree by these sera. Surprisingly, an anti-HLA antisera, anti-A_{11}, presumably unreactive with the lymphocyte donor haplotype, also showed significant blocking of Cx, α_H and β-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 ^3H 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 α_2 preparation in order to establish conditions for labeling. These results are summarized in Table 7.

At the highest ratios of protein (μl) to Iodogen (μg) 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

Table 7. Effect of iodogen levels and time of exposure on both the efficiency of labeling and on preservation of human α_2 lytic activity

Volume α_2 (μl)	Iodogen plated (μg)	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

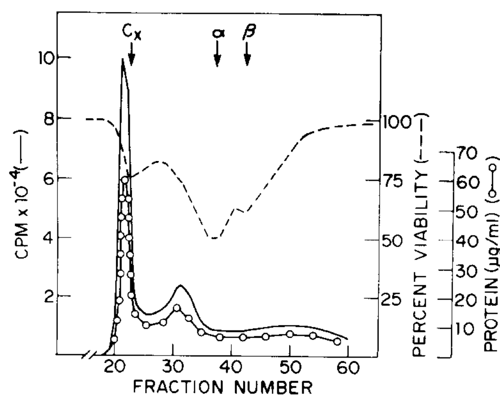

Fig. 3. Radiolabeled whole supernatant from PHA-stimulated adenoid lymphocytes resolved on Ultrogel AcA 44. Each fraction was tested for lytic activity on L cells (---), protein concentration by the fluorescamine assay (O——O), and radioactivity (——).

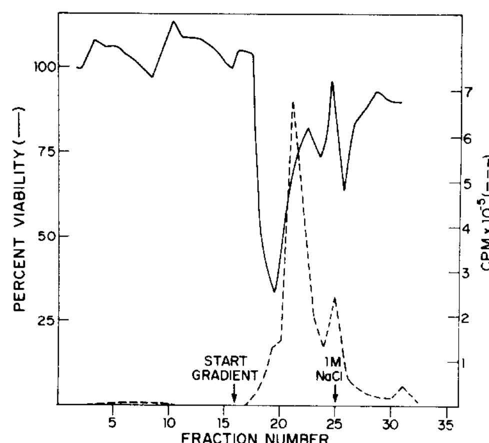

Fig. 4. Chromatography of radiolabeled human α_L lymphotoxin on DEAE-cellulose. The linear 0–0.3 *M* NaCl gradient was applied from fraction 16–25, and 1 *M* NaCl wash from 25–35. Each fraction was tested for lytic activity on L cells (——) and radioactivity (---).

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 1½ ml sample was iodinated, using 1 μg of Iodogen, and ~500 μCi ^{125}I. 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 α class LT on DEAE-cellulose. To ascertain the purification of α_L achieved by chromatography on DEAE-cellulose, 200 μl of α_L was iodinated with 1 μg of Iodogen and ~500 μCi ^{125}I with a 5 min

Table 8. Purification of α_L-LT

Source	LT activity (units)	Protein	Specific activity (units/μg protein)
1.5 ml Whole supernatant (50× concentrate)	α ≃1000	≃300	≃3
200 μl α_2 (10× concentrate) on DEAE-cellulose	α_2 ≃125	≃5 μg	≃25
50 μl α_2 on Con A-Sepharose	α_2 ≃25	≃200 ng	≃125
50 μl α_2 on PAGE after Con A-Sepharose	α_2 ≃20	≃20 ng	≃1000

reaction. After multiple dialysis changes against starting column buffer, the labeled α_L 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 μl) and radioactivity (100 μl). In Fig. 4, the peak of major lytic activity, α_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 α_2 preparation is overwhelmingly dominated by contaminating proteins (data not shown).

Purification of labeled α_2 on lectin columns. Previous studies from this laboratory (Toth & Granger, 1979) have shown that human α_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 α_2 and used Con A-Sepharose columns to purify the glycoprotein in the following manner: 50 μl of a 500 μl preparation of α_2, which had been iodinated with 3 μg 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 m*M* galactose in PBS, 200 m*M* methyl-glucopyranoside in PBS, and then 200 m*M* methyl-glucopyranoside in 0.5 *M* NaCl in PBS. The fractions were assayed for radioactivity (10 μl) and lytic activity (200 μl). The results are shown in Fig. 5.

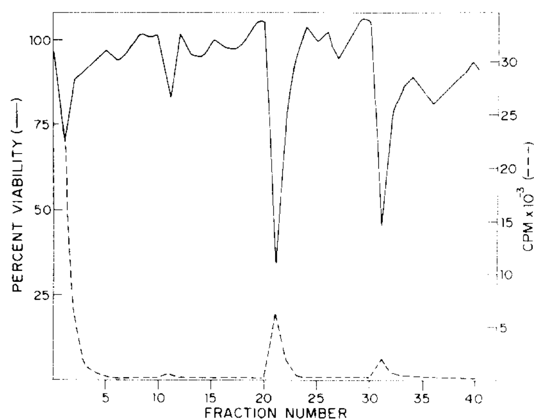

Fig. 5. Affinity chromatography of radiolabeled human α_2 lymphotoxin on Con A-Sepharose. The lymphotoxin preparation was labeled with Iodogen as described in Materials and Methods. The labeled preparation was applied to the lectin column equilibrated in PBS, and washed with this buffer. At fraction 11, a 200 m*M* galactose in PBS wash was begun. This was followed, at fraction 21, by specific desorption with 200 m*M* methyl-glucopyranoside in PBS, and then 200 m*M* methyl-glucopyranoside with 0.5 *M* NaCl in PBS. Each fraction was assayed for lytic activity (——) and radioactivity (---).

Most of the protein and some of the lytic activity ($\sim$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 $\sim$20% of the total protein applied. Further lytic activity ($\sim$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 α_2. In order to determine if the lectin affinity separation of α_2 resulted in a homogeneous product, we conducted the following experiment. Two hundred microliters of α_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 μg of Iodogen for 5 min with 500 μCi ^{125}I. After dialysis against 500 vol. PBS for 1 hr, 300 μl 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 μl of

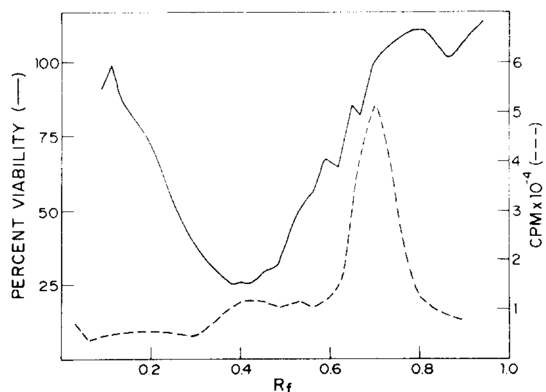

Fig. 6. PAGE of labeled Con A-Sepharose purified human α_2 lymphotoxin. Human α_2 was purified by lectin-affinity chromatography, labeled with Iodogen, and subjected to PAGE as described in Materials and Methods. Both the lytic activity on L cells (——) and radioactivity (---) in each gel slice were determined.

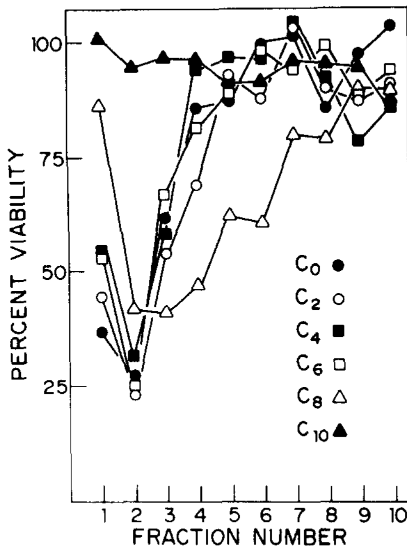

Fig. 7. Effect of alkyl chain length on adsorption of human α_2 to hydrophobic columns. Human α_2 was applied to each of a series of alkyl columns: C_0—unsubstituted Sepharose (●), C_2—ethyl Sepharose (○), C_4—*n*-butyl-Sepharose (■), C_6—*n*-hexyl-Sepharose (□), C_8—*n*-octyl-Sepharose (△) and C_{10}—*n*-decyl-Sepharose (▲). After allowing binding, the columns were developed with PBS, and the lytic activity in each fraction determined as indicated.

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 $R_f \sim 0.4$. Obviously, this is still a very impure preparation, which is dominated by a large protein peak at $R_f \sim 0.7$.

Hydrophobic chromatography of α_2. We have begun to explore the utility of hydrophobic chromatography in purifying lymphotoxins. Early work with the human α_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_0–C_{10} alkyl columns was loaded 100 μl of α_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 Cx and α_H LT forms appear to be comprised in part of the α_L, β and γ components, which may be dissociated through perturbation of weak, noncovalent bonds. An antigen-specific Cx 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 α_H 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 Cx and α_H 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 Cx and α_H 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

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 radioiodine 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 α_L 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 α_2 preparation, purified on Con A-Sepharose, as seen in PAGE ($R_f \sim 0.7$). 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 α_2 preparation, a labeled protein which comigrates with α_2 lytic activity in electrophoresis. This protein appears homogeneous in two sequential electrophoretic procedures. Experiments to verify further the identity of this protein with α_2 lymphotoxin molecules are under way in our laboratory.

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