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Authors

Johnson, Diane L
Yamamoto, Robert S
Plunkett, J Michael
[et al.](#)

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PURIFICATION TO ELECTROPHORETIC HOMOGENEITY OF HUMAN ALPHA LYMPHOTOXIN FROM A CLONED CONTINUOUS LYMPHOBLASTOID CELL LINE IR 3.4

Diane L. Johnson, Robert S. Yamamoto, J. Michael Plunkett, Irene K. Masunaka,
and Gale A. Granger

Department of Molecular Biology and Biochemistry, University of California, Irvine,
Irvine, California

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ABSTRACT

The 70-90,000 molecular weight (MW) alpha (α) component of the human lymphotoxin (LT) system has been purified to electrophoretic homogeneity. The α LT containing supernatants were obtained from a phorbol myristate (PMA) stimulated cloned continuous human B lymphoblastoid cell line IR 3.4. Supernatants were subjected to a biochemical separation scheme that consisted of chromatography on control pore glass beads, DEAE ion-exchange chromatography, lentil-lectin affinity chromatography, and electrophoresis on 7% native preparative polyacrylamide gels. The specific activity of the α LT in the final fractions was from 10^7 to 5×10^7 units of LT activity/mg protein. Approximately 3 to 5% of the initial α LT was recovered in the final fractions and a purification factor of 25,000 to 30,000 fold was required to achieve homogeneity. The α LT preparation from preparative PAGE exhibited coincident migration of bioactivity and radioactivity on 5 and 7% native PAGE tube gels. Only a single protein peak was observed when the radiolabeled α LT was subjected to a two-dimensional SDS-reducing slab gel.

MATERIALS AND METHODS

Phorbol myristate acetate (PMA) mitomycin-C, ethylene glycol, and α -methyl-D-mannoside (α MM) (Sigma, St. Louis, MO); fetal calf serum (FCS), lactalbumin hydrolysate (LAH), and RPMI-1640 (GIBCO, Grand Island, NY); controlled pore glass beads (CPG-240) and iodogen (Pierce, Rockford, IL); Whatman DE-11 resin (Maidston, Kent, England); Ultrogel AcA 44, ampholines (LKB, Bromma, Sweden); lentil-lectin Sepharose-4B (Pharmacia, Piscataway, NJ).

The cloned continuous human B lymphoblastoid cell line, IR 3.4 were grown to a cell density of 10^6 to 1.5×10^6 cells/ml in RPMI-1640 plus 10% FCS (Yamamoto et al, 1983). Washed cells were resuspended in RPMI-1640 supplemented with LAH at 0.1% as a serum substitute and cultured in the presence of 20 ng/ml PMA for three days (Yamamoto et al, 1983). After this culture period, the supernatants were cleared of cells by centrifugation.

The assay for LT activity was conducted as described previously (Granger et al, 1978). Column fractions or gel slices which had been eluted overnight in RPMI-1640 plus 3% FCS were applied in a volume of 50-200 μ l to a monolayer of 10^5 mitomycin-C treated L-929 cells cultured for 24 hrs in 1 ml of RPMI-1640 plus 3% FCS. After an additional 16-24 hr incubation, the remaining viable adherent cells were determined employing a Coulter Counter.

Supernatants were first subjected to chromatography on a CPG-240 column. Supernatants (1 L batches) were passed through a 20 ml CPG-240 column at a flow rate of 50 ml/hr at 4°C. Bound material was desorbed with 100 mls of 50% ethylene glycol in a 10 mM Tris, 1 mM EDTA, pH 8.0 buffer (Tris buffer) at a flow rate of 25 ml/hr at 25°C. All fractions were assayed for lytic activity and monitored for protein using a Bio-Rad Protein Assay Kit 1 (Bio-Rad, Richmond, CA).

One hundred mls of the CPG-240 desorbed sample was loaded on a 2.5 X 4.5 cm DEAE column equilibrated in Tris buffer. The LT sample was eluted with a linear gradient of NaCl from 0.05 to 0.3 M Tris buffer and protein, conductivity, and lytic activity were measured as previously described (Granger et al, 1978).

The α LT from the DEAE column was pooled and next applied directly to a 3.3 X 1.0 cm lentil-

lectin column equilibrated in Tris buffer. The α LT was passed through the column at a flow rate 12 mls/hr at 4°C. The column was washed with Tris buffer and the bound sample was then desorbed with 40 mls of 200 mM α MM in Tris buffer. One ml fractions were collected at a flow rate of 12 ml/hr and assayed for lytic activity and protein.

The LT activity eluted from the lentil-lectin column was concentrated, and radioiodinated employing the iodogen technique (Fracker and Speck, 1978; Klostergaard *et al.*, 1981a). Labeled material was subjected to preparative electrophoresis on 7% native polyacrylamide gels (PAGE) (Furlong *et al.*, 1973; Klostergaard *et al.*, 1981b). Twenty drop fractions were collected as material ran off the gel at a flow rate of 120 drops/hr and was immediately assayed for radioactivity and lytic activity. The fractions containing lytic activity were pooled, concentrated, reiodinated, and subjected to 5 and 7% native PAGE tube gels by the technique of Davis (1964). Each gel slice was assayed for radioactivity and lytic activity (Klostergaard *et al.*, 1981a).

The radiolabeled α LT activity eluting from the preparative PAGE columns was also subjected to two-dimensional electrophoresis by the method of O'Farrell (1975). The first dimension employed isoelectric focusing in 3 X 70 mm borsilicate tubes with amphaline 0.4%, pH 3-10; 0.8%, pH 4-6; and 0.8%, pH 6-8. After equilibrating the focused gel in denaturing sample for 30 minutes, the gel was loaded on a 15% SDS 13 X 14 X 0.8 cm slab gel and electrophoresed at 20 mA. Gels were dried and autoradiographed by XRP-5 X-OMAT film (Eastman Kodak, Rochester, NY) with Cronex Lighting Plus intensifying screens (Dupont, Claremont, CA).

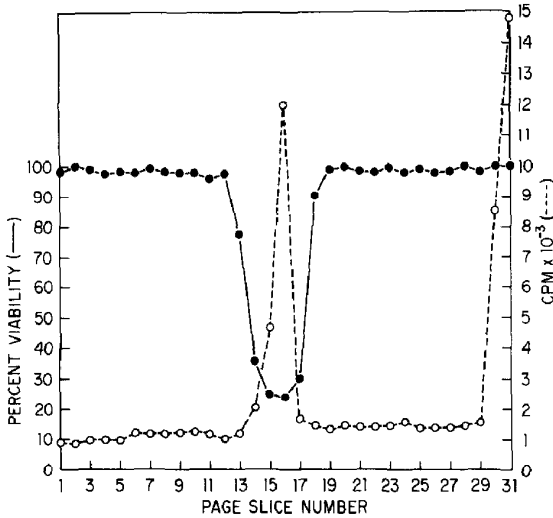
RESULTS

Supernatants from PMA activated IR 3.4 cells were first subjected to chromatography on CPG-240 columns. Analysis of the fractions revealed that 85 to 90% of the lytic activity and 28 to 32% of the protein was adsorbed by the CPG-240 column. The amount of lytic activity desorbed from the CPG-240 column was 20% of that in the original starting supernatant. However, this represents 30 to 50% of the activity in this supernatant which is due to the α molecule. The elutant contained from 9 to 14% of the total protein detected in the initial supernatant.

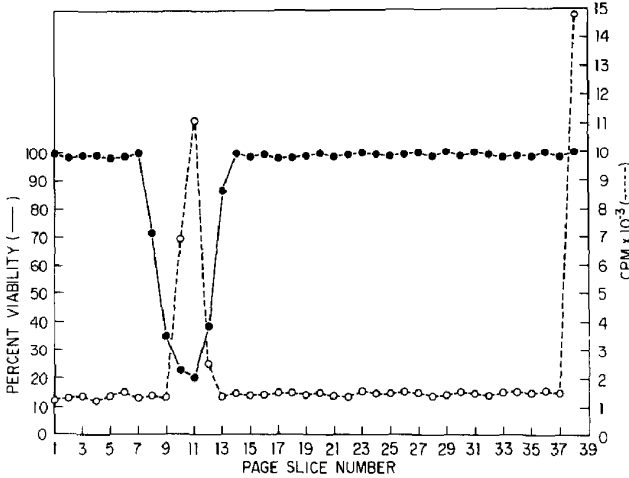
The lytic material desorbed from the CPG-240 column was next applied and eluted from a DEAE ion exchange column. From 10 to 20% of the lytic activity routinely did not bind to the DEAE column. Immunological and biochemical studies revealed this activity was not due to α LT forms but due to an unrelated β LT form. The remaining lytic activity, 40-50% eluted as a sharp single major peak at 0.03 M NaCl while the majority of the protein eluted as a broad peak at 0.15 M NaCl. This material is all the α form as judged by its reactivity with anti- α serum and elution from molecular sieving columns.

The α peak of lytic activity from the DEAE column was next applied to a lentil-lectin column. The lentil-lectin column adsorbed from 92 to 98% of the lytic activity applied to the column. The α MM buffer desorbed 40 to 45% of the lytic activity and 3 to 11% of the protein applied to the column.

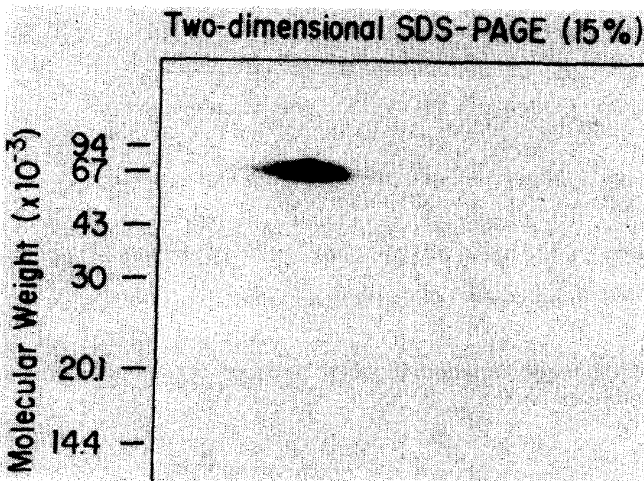
The lytic activity desorbed from the lentil-lectin column was pooled, concentrated, and radioiodinated. The radiolabeled material was electrophoresed on a 7% native PAGE tube gel. The gels were cut into 1 mm slices and counted for radioactivity and then eluted with RPMI-1640. The results of this study indicate that the lentil-lectin sample contained multiple components but a good separation of lytic material (Rf 0.32) and nonlytic material (Rf 0.84) was achieved so the final separation of the lentil-lectin material could be achieved on a high capacity preparative native PAGE column. The bioactivity from the lentil-lectin sample was radioiodinated, electrophoresed, and eluted from the preparative PAGE column. The bioactivity eluted from the preparative PAGE was re-labeled with ^{125}I and rerun on 5 and 7% native PAGE tube gels. The data shown in Figure 1 illustrates that both radioactivity and bioactivity migrates as a sharp single coincident peak in both 5% (upper panel) and 7% (lower panel) PAGE tube gels. The radiolabeled LT was next analyzed on a two-dimensional O'Farrell gels. Figure 2 shows the autoradiograph of one of these gels and it is evident that a single 68,000 MW peptide is present in the sample. However, no bioactivity could be recovered from these gels because the SDS at the concentration used abolished all detectable lytic activity. Additional studies revealed the material obtained from the preparative PAGE column elutes from molecular sieving on Ultrogel AcA 44 as a 70-90,000 MW α LT class.



Analytical discontinuous PAGE (5% acrylamide) of IR 3.4 α LT by preparative PAGE. Radioactivity (O) and lytic activity (·) of each gel slice was determined.



Analytical discontinuous PAGE (7% acrylamide) of IR 3.4 α LT by preparative PAGE. Radioactivity (O) and lytic activity (·) of each gel slice was determined.



Two dimensional 0.1% SDS 15% acrylamide slab gel of IR 3.4 α LT by preparative PAGE. Molecular weight markers were phosphorylase B (94,000 MW), BSA (67,000 MW), ovalbumin (43,000 MW), carbonic anhydrase (30,000 MW), soybean trypsin inhibitor (20,000 MW), and lysozyme (14,400 MW).

DISCUSSION

This report documents the purification of α LT from a PMA activated continuous B cell line to electrophoretic homogeneity. The data for complete purification runs for two supernatant lots are shown in Table 1. There is a 5500 to 6500 fold purification after the lentil-lectin step with approximately 3% recovery of the original LT activity of the whole supernatant. The preparative PAGE column removed about 80% of the contaminating radiolabeled protein resulting in an additional 5 fold purification. The final step resulted in a 25,000 to 30,000 fold purification of the α LT. An exact calculation of the specific activity of the α LT in the final fractions is not possible because of the fluctuation in the bioassay itself. However, specific activity ranged from a low of 10^7 to a high of 5×10^7 units/mg protein. The α LT from the IR 3.4 cell line appears to be a 68,000 MW peptide which is in agreement with the α LT molecule purified from lectin-stimulated normal human lymphocytes (Klostergaard *et al.*, 1981a), and α LT immunoprecipitated from the human Cx LT form by anti- α serum (Devlin *et al.*, submitted for publication). The availability of the α LT in a pure form will allow both functional and structural studies of this interesting lymphokine.

Table 1. Biochemical Purification Scheme of the IR 3.4 α LT

Separation Method	Supernatant Lot	Percent Recovery of LT Units ^{a)}	Protein	
			% Recovery ^{b)}	Purification Factor ^{c)}
CPG	1	18	9	11.11
	2	22	14	7.15
DEAE	1	7.5	0.58	172.41
	2	7.6	0.15	666.67
Lentil-Lectin	1	3.0	0.015	6666.67
	2	3.4	0.017	5882.35
Preparative PAGE	1	1.5	0.003	33333.34
	2	2.0	0.0034	29411.75

^aPercent recovery of LT from the original starting IR 3.4 supernatant as calculated by the formula: [(Total LT units after biochemical step)/(Total LT units from unseparated supernatant)] X 100.

^bPercent recovery of protein from the original starting IR 3.4 supernatant as calculated by the formula: [(Total protein after biochemical step)/(Total protein from unseparated supernatant)] X 100.

^cPurification factor as calculated by the formula: (Percent starting protein)/(Percent recovery).

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