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PRELIMINARY COMMUNICATION

HUMAN LYMPHOTOXINS: PURIFICATION TO ELECTROPHORETIC HOMOGENEITY OF THE α_H RECEPTOR-BEARING CLASS

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ABSTRACT

The α_H component (120-150,000 d) of the human LT system has been purified to electrophoretic homogeneity. This form was obtained from Con A-stimulated human tonsil and adenoid lymphocytes. Purification was greatly facilitated by employing a low concentration of lactalbumin hydrolysate as a serum substitute. The scheme consisted of sequential molecular sieving, lectin-affinity chromatography on Con A-Sepharose, and isoelectricfocusing. The specific activity of the α_H increased approximately 5-10,000-fold in the course of purification, as judged by protein monitoring by the fluorescamine assay and radioiodination using the Iodogen technique. Homogeneity of the α_H preparation from electrofocusing was demonstrated by native PAGE, as only a single protein peak was observed. The identity of this radioactive peak with the lytic moiety was apparent as it migrated to the only region of the gel where lytic activity was detectable. This represents the third human lymphokine purified to homogeneity.

MATERIALS AND METHODS

Concanavalin A (Con A) and mitomycin C (Sigma, St. Louis, MO); newborn calf serum, lactalbumin hydrolysate (LAH), and RPMI-1640 (Gibco, Grand Island, NY); Ultrogel AcA 44 and ampholines (LKB, France); Con A-Sepharose (Pharmacia, Piscataway, NJ); methyl-α-D-glucopyranoside (MGP) (Calbiochem, La Jolla, CA); fluorescamine (Aldrich, Milwaukee, WI).

Human tonsil and adenoid lymphocytes were prepared as single-cell suspensions as previously described (Lewis, et al., 1976). These cells were suspended in RPMI-1640, using LAH at 0.01% as a substitute for serum (Klostergaard, et al., in preparation). After stimulation with 10 μg/ml Con A for five days, the cells were cleared from their supernatants. The supernatants were concentrated 50X employing a Hollow-fiber Filter Concentrator (Amicon, Lexington, MA).

These concentrates were subjected to molecular sieving on Ultrogel AcA 44, using Blue Dextran, IgG, hemoglobin, and phenol red as molecular weight markers. Fractions were tested for lytic activity (see below) and protein using the fluorescamine assay with a BSA standard (Klostergaard, et al., 1980).

The α_H peak from molecular sieving was pooled, concentrated and radioiodinated employing the Iodogen technique (Fraker and Speck, 1978; Klostergaard, et al., 1980). After dialysis, the labeled α_H was applied to Con A-Sepharose equilibrated in PBS (10 mM phosphate, 0.15 M NaCl, pH 6.9). After washing with PBS, a 0-200 mM linear gradient of MGP in PBS was applied. Lytic activity (see below) and radioactivity in each fraction was determined.

The Con A-purified ^{125}I-α_H was subjected to isoelectrofocusing in a Type 8101 Ampholine Column (LKB). A gradient from pH 4-8 in sucrose was employed. After focusing for 16 hr at a final voltage of 800 v, the column was drained, collecting 60 drop fractions. Each fraction was tested for radioactivity, and alternate fractions were dialyzed against 100 volumes of PBS for 4-16 hr and then tested for lytic activity (see below).

Native polyacrylamide gel electrophoresis (PAGE) of α_H at different stages of purification was performed as previously described (Davis (1964), Klostergaard, et al. (1980)).

Running gel acrylamide concentration was 5 or 7%. Each gel slice was assayed for radioactivity and lytic activity (see below).

Column fractions or gel slices which had been eluted overnight in media (RPMI-1640) containing 0.1% LAH were applied in a volume of 50-200 µl to a monolayer of 10^5 mitomycin-C treated α-L-929 cells cultured for 24 hr in 1 ml of RPMI-1640 containing 3% newborn calf serum. After 16-24 hr incubation, the remaining viable adherent cells were determined employing a Coulter counter.

RESULTS

Concentrated supernatants from Con A-activated human tonsil and adenoid lymphocytes were subjected to molecular sieving on Ultrogel AcA 44. Assay for lytic activity revealed that the major (>80%) peak of lytic activity was in the 120-150,000 d range, termed α_H lymphotoxin. The fluorescamine assay indicated two major biosynthetic protein peaks, in the void volume and near 150,000 d, and a large trailing peak near the phenol red marker, probably reflecting residual LAH.

The α_H peak from molecular sieving could be significantly purified on Con A-Sepharose. From 80-90% of the α_H bound the adsorbent, while ~98% of the radioactivity appeared in the breakthrough fractions; the binding could be reversed with MGP. Thus, a 50-fold increase in specific activity, with high yield, was realized by lectin-affinity chromatography of α_H on Con A-Sepharose.

This purified preparation was shown to still be heterogeneous by native PAGE analysis (Fig. 1). Electrophoresis in a 7% gel (upper panel) shows a major broad peak of lytic activity (R_f ~0.25), and a minor peak with somewhat higher R_f (~0.5). Only a shoulder of the major radioactive peak, centered at R_f 0.35 corresponds to the center of the major lytic peak. Electrophoretic analysis of the same preparation in a 5% gel (middle panel) reveals a small (~5% of total counts) radioactive peak (R_f ~0.35) corresponding to the major lytic activity; the broad lytic peak observed in the 7% gel has in turn been resolved into peaks at R_f 0.35 (major), and 0.5 (minor), in the 5% gels.

Since PAGE analysis indicated that the lectin-purified α_H was dominated by proteins more acid than the predominant lytic moiety itself, this preparation was subjected to further purification by isoelectricfocusing (Fig. 2). Resolution on a pH 4-8 gradient revealed that the major lytic activity focused broadly in the 6-7 region (Fractions 38-52, Fig. 2). The radioactive profile indicated major protein peaks at pI's lower than this range, while only a trough corresponded to the lytic fractions. The fractions containing the lytic activity were pooled into four distinct pools, each covering a quarter of the region from pH 6-7 (fractions 40-42, 43-45, 46-48, 49-51, respectively). These pools were concentrated and analyzed by native PAGE. The lower panel of Figure 1 shows a representative electrophoretogram for one of these pools (fractions 43-45) in a 7% acrylamide gel. Only a single, sharp radioactive peak (R_f ~0.25), corresponding well with the center of the broad, major lytic peak (upper panel) is observed. All the more rapidly migrating contaminants present in the Con A purified α_H (upper panel) have been eliminated by electrofocusing. Therefore, the predominant species of human α_H lymphotoxin appears to have been purified to homogeneity by this scheme.

DISCUSSION

Human T-enriched lymphocytes and MLC lymphoblasts, when stimulated with lectin, release cell-lytic molecules which are associated with antigen-binding receptor(s) (Harris, et al. (1981b); Ware, et al. (submitted)). The evidence to suggest the presence of receptor(s) is derived from both functional and serological studies. Biochemical studies have shown that the receptor-bearing lymphotoxin classes are the Complex (>200,000 d) and alpha-heavy (120-150,000 d) (Hiserodt, et al. (1978a, 1978b); Harris, et al.(1981b)). These forms, in addition to being receptor-directed, are capable of fairly rapid (8-12 hr) lysis of a spectrum of allogeneic target cells (Yamamoto, et al. (1979); Harris, et al. (1981b)).

Studies designed to ascertain the role of these T-cell derived, cytotoxic, receptor-associated molecules in in vitro models of cell-mediated immunity have been impaired by their lability as well as by the complexity of the supernatant in which they are found. The availability of these molecules in a stable purified form would facilitate the understanding of cellular and molecular mechanisms in which they participate.

This report documents the purification to homogeneity of α_H lymphotoxin from lectin-stimulated nonimmune lymphocytes. This form appears to be a T-cell product, since it reacts with a heterologous anti-human $F(ab')_2$ antiserum, as do the α_H forms from human MLC blasts and T-enriched lymphocytes. This reactivity is not attributable to the presence of classical Ig determinants, since a battery of anti-human heavy- and light-chain sera are without marked effect (Klostergaard, et al., in preparation).

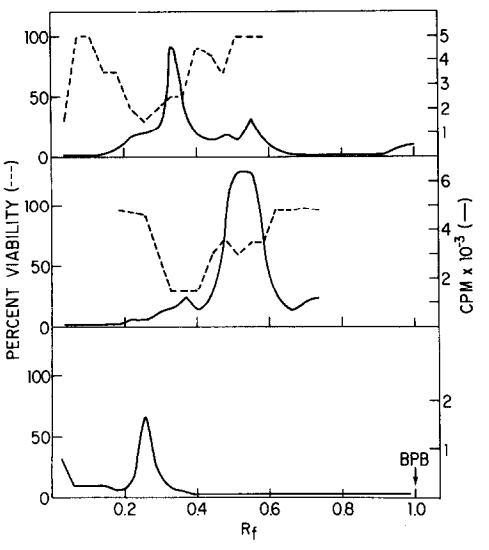

Fig. 1 Polyacrylamide gel electrophoretograms (PAGE) of ^{125}I-α_H.

Upper panel - Analysis in a 7% gel of ^{125}I-α_H purified by molecular sieving and lectin-affinity chromatography on Con A-Sepharose. Radioactivity (——) and lytic activity (-----), as determined by loss of viable adherent cells from an α-L-929 cell monolayer, present in gel slices is shown.

Middle panel - Analysis in a 5% gel of the same preparation as in upper panel.

Lower panel - Analysis in a 7% gel of ^{125}I-α_H purified by molecular sieving, lectin-affinity chromatography, and isoelectricfocusing. Fractions 43-45 (Fig. 2) were pooled and concentrated and subjected to PAGE.

The purification scheme utilizes serum-free media, which facilitates the purification of this mediator which is probably present in supernatants at the nanogram per milliliter range. We have employed lactalbumin hydrolysate as a serum substitute; therefore the residual exogenous protein is readily removed by molecular sieving as it elutes very much later than the α_H lytic peak (120-150,000 d). Furthermore, the α_H derived from lactalbumin hydrolysate-containing supernatants is more stable than that derived from serum-containing cultures (Harris, et al. (1981a), Klostergaard, et al., in preparation).

The purification scheme is comprised of three tandemized standard biochemical procedures: molecular sieving, achieving five-to-tenfold purification; lectin affinity chromatography, which raises the specific activity about 50-fold; and isoelectricfocusing, which entails a purification factor of about 20-fold. Thus, the overall increase in specific activity of the homogeneous product is 5-10,000-fold, despite starting with very low protein supernatants. This underscores the difficulty encountered by many investigators in purifying factors from lymphocyte supernatants. The yield for each step is 70-80%, thus giving an overall yield of ~40%. Considerable microheterogeneity is apparent in native PAGE as well as in electrofocusing; most of the lytic activity focuses in the 6-7 pI range, although minor components are seen with lower pI's (Fig. 2). Despite this microheterogeneity, PAGE analysis reveals that there are no non-lytic contaminants present in the pH 6-7 range (Fig. 1), which corresponds to the major lytic component.

The availability of the α_H lymphotoxin molecule in a pure form will allow resolution of its peptide substructure. Peptides bearing lytic function and receptor function will be identified by affinity methods employing specific antisera in SDS gel systems and by partial amino acid sequencing. These peptides will be compared to the pure human α_L lymphotoxin class (Klostergaard, et al. (submitted) at the level of peptide mapping. Specific antisera to the pure α_H class are being raised and will be used to probe blocking various in vitro cell-mediated cytotoxic reactions. Finally, other lymphokine activities expressed by the α_H molecule are being determined in collaboration with other laboratories.

REFERENCES

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

2. Fraker, P. J., and Speck, J. C. (1978) Protein and cell membrane iodinations with a sparingly soluble chloramide, 1,3,4,6-tetrachloro-3α,6α-diphenyl-glycoluril. Biochem. Biophys. Res. Commun., 80, 840.

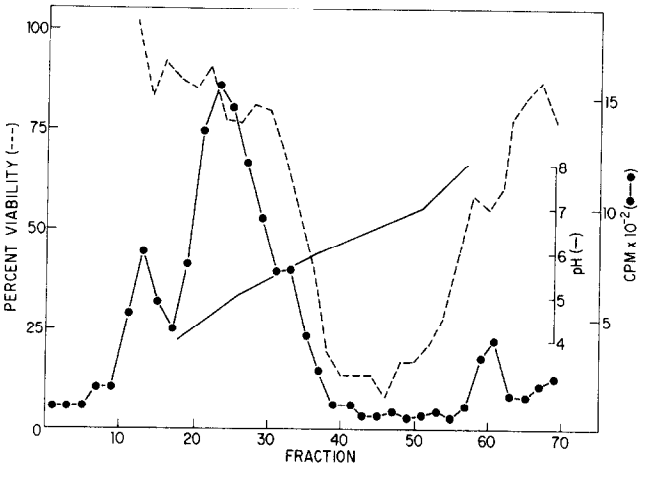

Fig. 2

Isoelectricfocusing of ^{125}I-α_H purified by molecular sieving and lectin-affinity chromatography on Con A-Sepharose. The pH of alternate fractions was tested to establish the gradient; in the other fractions, radioactivity was determined, and after dialysis to eliminate sucrose and ampholines, these fractions were assayed for lytic activity. The region containing the major lytic activity (Fractions 40-51) was split into discrete pools and concentrated (see text). The pool from fractions 43-45 was analyzed by PAGE (Fig. 1, lower panel).

3. Harris, P. C., Yamamoto, R. S., Crane, J., and Granger, G. A. (1981a) The Human LT System X: The initial form released by T-enriched lymphocytes is 150,000 MW, associated with small non-lytic components, and can dissociate into the smaller α, β, and γ MW classes. J. Immunol. (in press).

4. Harris, P. C., Yamamoto, R. S., Christensen, C., and Granger, G. A. (1981b) The Human LT System XI: Cell-lytic effectiveness and association of certain LT forms with Ig-like antigen binding receptor(s) released by T-enriched lymphocytes. J. Immunol. (in press).

5. Hiserodt, J. C., Yamamoto, R. S., and Granger, G. A. (1978a) The Human LT System III. Characterization of a high molecular weight LT class (Complex) composed of the various smaller MW LT classes and subclasses in association with Ig-like molecules. Cell. Immunol. 38, 417-433.

6. Hiserodt, J. C., Yamamoto, R. S., and Granger, G. A. (1978b) The Human LT System IV. Studies on the large MW LT Complex class: Association of these molecules with specific antigen binding receptor(s) in vitro. Cell. Immunol. 41, 380-396.

7. Klostergaard, J., Yamamoto, R. S., and Granger, G. A. (1980) Human and murine lymphotoxins as a multicomponent system: Progress in purification of the human α_L component. Molec. Immunol. 17, 613-623.

8. Lewis, J. E., Yamamoto, R. S., Carmack, C., Lundak, R. L., and Granger, G. A. (1976). Lymphocyte effector molecules: An in vitro production method for obtain liter volumes of supernatants from mitogen-stimulated human lymphocytes. J. Immunol. Meth., 11, 371-383.

9. Yamamoto, R. S., Hiserodt, J. C., and Granger, G. A. (1979) The Human LT System V: A comparison of the relative lytic effectiveness of various MW human LT classes on ^{51}Cr-labeled allogeneic target cells in vitro: Enhanced lysis by LT complexes associated with Iglike receptor(s). Cell. Immunol., 45, 261-275.