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PURIFICATION OF PRIMATE T CELL GROWTH FACTOR TO APPARENT HOMOGENEITY BY REVERSE PHASE HIGH PRESSURE LIQUID CHROMATOGRAPHY: EVIDENCE FOR TWO HIGHLY-ACTIVE MOLECULARLY-DISTINCT SPECIES

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SUMMARY: T cell growth factor produced by the MLA144 gibbon ape lymphosarcoma T lymphocyte line was separated into two molecular forms of M_r 16,300 and M_r 14,300 which were purified 32,000-fold and 82,000-fold, respectively, and which in combination constitute the majority of biological activity of the starting material. The overall recovery of biological activity was 50%. This was accomplished with a series of chromatographic steps including reverse-phase high pressure liquid chromatography. The purified proteins are of comparable specific activities and each maintains the DNA synthesis of T cell growth factor-dependent human T cell lines at concentrations of less than approximately 18 pM (300 fg/ml).

T cell growth factor (TCGF[1], also known and Interleukin 2), first described by Gallo and his colleagues in 1976 (1), is a protein produced by normal T lymphocytes of many species upon stimulation by lectins or antigens (2). TCGF is mitogenic for activated T lymphocytes (2) and may thus be essential for the expansion of antigen-specific precursors to form functional populations of helper, suppressor and cytotoxic T lymphocytes. As has been noted by others (3,4), unpurified or partially purified preparations of TCGF contain interfering substances which prevent precise delineation of the structure and mechanism of action of this lymphokine. In this study, we present conclusive evidence for the existence of two molecularly distinct species of TCGF of similar specific activity obtained in highly purified forms

[1] Abbreviations: TCGF, T cell growth factor; Na/K P_1, Na/K phosphate buffer pH 7.4; GF-HPLC, gel filtration high pressure liquid chromatography; RP-HPLC, reverse phase high pressure liquid chromatography; TFA, trifluoroacetic acid; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; 2-ME, 2-mercaptoethanol.

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which comprise the majority of TCGF produced by MLA144 (9), a gibbon ape lymphosarcoma T lymphocyte line.

MATERIALS AND METHODS

Production of TCGF. MLA144 cells were maintained as described by Rabin et al (9) and expanded to large numbers in spinner cultures. For TCGF production the cells were placed in spinner cultures at 1.7 X 10^6 cells per ml without serum (3.5 to 4.0 liters) for 72 hr at 37°C. Conditioned medium containing TCGF was harvested by centrifugation at 1,000 x g for 30 min at 4°C.

Concentration steps. Four liters of conditioned medium was adjusted to pH 7.2 with 1 N NaOH and applied to two 50 ml columns of hydroxylapatite (HTP Grade, Bio-Rad, Richmond, CA) equilibrated in 5 mM Na/K phosphate buffer, pH 7.4 (Na/K P_i). TCGF was eluted with 90 ml/column each of 85 mM and 115 mM Na/K P_i, pooled, supplemented with polyethylene glycol-8000 (PEG-8000) to a final concentration of 0.1% (w/v) and dialyzed vs 50 mM NaCl, 0.1% (w/v) PEG-8000, 5 mM Na/K P_i (dialysis buffer). The unbound material from two 40 ml columns of DEAE-cellulose (DE52, Whatman, England) equilibrated in dialysis buffer contained the TCGF activity and was applied to a 10 ml hydroxylapatite column equilibrated with dialysis buffer, eluted with 15 ml of 400 mM Na/K P_i, 0.1% (w/v) PEG-8000 and concentrated to between 0.5 and 1.0 ml by ultrafiltration using Amicon stirred cells and YM5 membranes.

Gel Filtration HPLC (GF-HPLC). GF-HPLC was performed on ultrafiltered material using a Varian 5000 series HPLC system, UV-50 absorbance detector, and a TSK G2000 SW column (7.5 X 500 mm) equilibrated with 150 mM NaCl, 0.3% (w/v) PEG-8000, 10 mM Na/K P_i at a flow rate of 0.5 ml/min. The Amicon concentrate was applied in 100 µl aliquots, each containing 2 to 3 mg of total protein.

Reverse-Phase HPLC (RP-HPLC). All reverse-phase HPLC was performed on Waters µ-Bondapak C_{18} columns (3.9 X 300 mm) using the HPLC system described above. The gel filtration fractions containing TCGF activity were pooled and applied to a column equilibrated in H_2O:-trifluoroacetic acid (TFA), 100:0.5 (v:v), the pH of which had been adjusted to 3.0 with NH_4OH. Proteins were eluted at a flow rate of 0.3 ml/min with a n-propanol gradient of 0.3%/min in the same buffer. Two peaks of TCGF biological activity were obtained, the first of which was diluted to a final n-propanol concentration of 10% and applied to a second C-18 column equilibrated in H_2O with sufficient TFA to lower the pH to 2.2 (~0.05-0.10%, v:v). Proteins were eluted as described above at pH 2.2.

Assay for TCGF Biological Activity. TCGF was assayed by a modification of the costimulator assay described by Shaw et al (5). A/SN strain mice (4-10 weeks of age) were kindly supplied by Dr. Donald C. Shreffler. Titers of TCGF were determined by serial dilution of samples and comparison to a crude MLA144 conditioned media standard arbitrarily assigned a concentration of 1 unit of TCGF/ml.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed as described by Laemmli in 15% acrylamide, 1.0 mm thick slab gels (6). Proteins were visualized by a silver staining technique (7). M_r was calibrated using standard proteins.

Protein Determination. Protein was assayed by a modification of the technique described by Lowry (8). The protein content of the RP-HPLC fractions was estimated by performing SDS-PAGE and comparing the silver staining intensity of the proteins in these fractions with the staining intensity of known amounts of protein standards.

RESULTS AND DISCUSSION

The MLA144 cell line used here constitutively produces TCGF titers comparable to those obtained from lectin activated single-donor human peripheral blood lymphocytes (9) but avoids potential stimulus artifacts of the

TABLE I
Summary of Purification of TCGF

	TCGF Units	Total Protein, µg	Specific Activity*	Purification per step	Purification overall	% Yield per step	% Yield overall
Starting CM	3810	~ 128,100	0.030	--	--	--	--
1st HA+	3070	67,800	0.045	1.5	1.5	81	81
DE52	2420	16,100	0.15	3.3	5.0	79	64
2nd HA+	4380	14,800	0.30	2.0	10	181	115
GF-HPLC	3210	1,900	1.71	5.7	57	73	84
1st RP-HPLC							
TCGF-I	1610	2.95	545	319	18,300	50	42
TCGF-II	907	~ 0.37	~2,450	1430	~ 82,300	28	24
2nd RP-HPLC							
TCGF-I++	635	~ 0.64	~1,000	1.8	33,600	64	27

* Units TCGF per µg protein
\+ HA = hydroxylapatite
++ 1000 units of TCGF-I from the first RP-HPLC column was applied to the second RP-HPLC column.

latter (11). After ultrafiltration the TCGF originally present in 4 liters of conditioned medium was present in a volume of 0.5-1 ml, the recovery of biological activity was apparently quantitative and a 10-fold increase in specific activity of TCGF had been achieved. (Table I). The total recovery of TCGF biological activity from the second hydroxylapatite column has consistently been greater than 100%. This probably reflects removal of substances which are inhibitory in the costimulator assay. When this material was applied to GF-HPLC (Fig. 1) TCGF eluted with a retention volume corresponding to a globular protein of M_r 23,600 (range 18,600-30,900). This is consistent with the results of Rabin et al (9) for MLA144-derived TCGF.

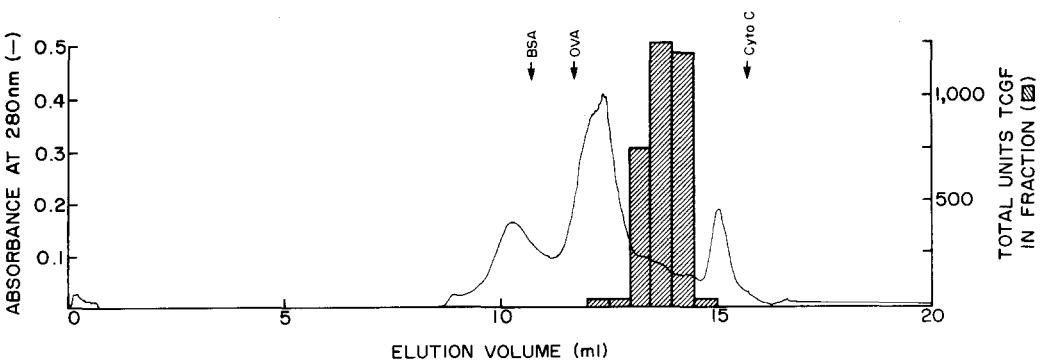

Figure 1: GF-HPLC of TCGF on TSK G2000SW. A 100µl aliquot of TCGF eluted from the second hydroxylapatite column and concentrated by ultrafiltration was fractionated in 150 mM NaCl, 0.3% (w/v) PEG-8000, 10 mM Na/K P_i, pH 7.4 at a flow rate of 0.5 ml/min. The column was calibrated with bovine serum albumin (BSA, M_r 68,000), ovalbumin (OVA, M_r 45,000) and cytochrome C (cyto C, M_r 12,500).

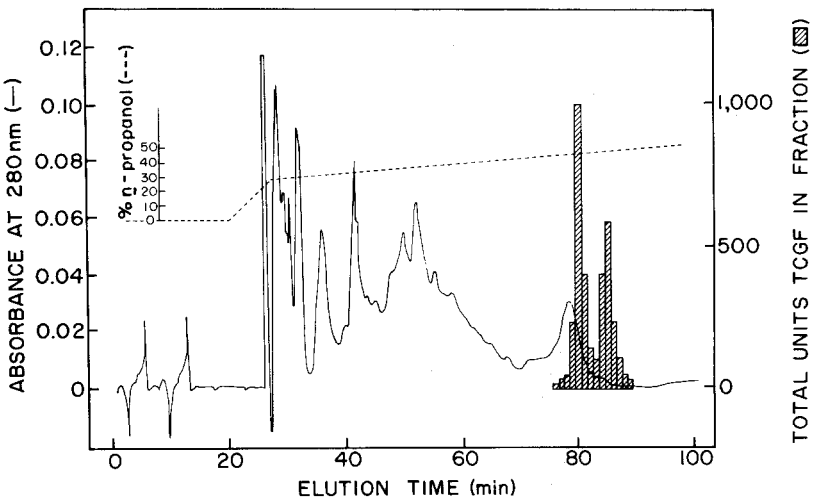

Figure 2: RP-HPLC of TCGF. TCGF eluting from GF-HPLC between 13.0 and 14.5 ml (figure 1) was fractionated on a μBondapak C_{18} column as described in the text using a mobile phase containing 0.5% (v/v) TFA buffered with NH_4OH to pH 3. (- - -) % n-propanol. One-minute fractions were collected.

When TCGF purified through GF-HPLC was applied to the first RP-HPLC column, two peaks of biological activity were resolved (Fig 2). The early peak elutes at 44% propanol and is referred to as TCGF-I. The later peak elutes at 45% propanol and is referred to as TCGF-II. When each peak was pooled separately and re-chromatographed in the identical RP-HPLC system single peaks of biological activity were obtained but the difference in elution time between the two species was maintained (unpublished data). SDS-PAGE in the absence of 2-mercaptoethanol (2-ME) demonstrated the presence of two proteins of apparent Mr 16,300 and 14,300 whose staining intensity correlated very well with the quantity of biological activity present in TCGF-I and TCGF-II, respectively (Fig 3a). TCGF-II did not contain other protein bands whose staining intensity correlated with biological activity suggesting that the M_r 14,300 protein is the active species. Since other bands were present together with the M_r 16,300 protein in TCGF-I, this material was subjected to a second RP-HPLC purification step with a mobile phase of lower TFA concentration. SDS-PAGE in the absence of 2-ME revealed a predominant (>90% of the total as judged by the intensity of staining) protein of apparent Mr 16,300 and a minor (<10%) protein of an apparent Mr 32,000 (unpublished data). SDS-PAGE in the presence of 2-ME

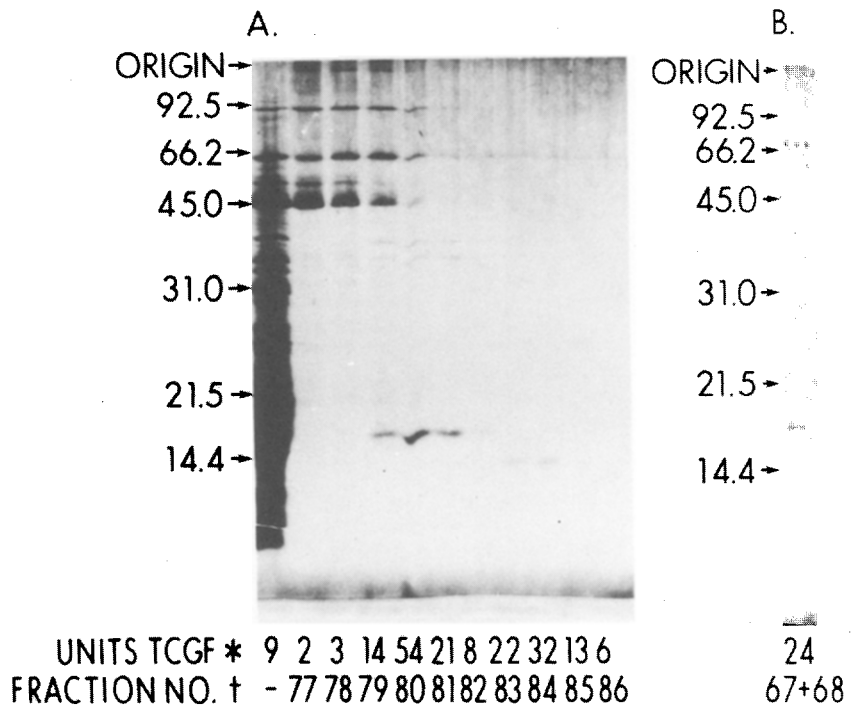

Figure 3: SDS-PAGE of TCGF purified by RP-HPLC. a) SDS-PAGE in the absence of 2-ME. The first lane is TCGF purified by GF-HPLC. Additional lanes are TCGF purified by a single RP-HPLC step (from figure 2). b) SDS-PAGE in the presence of 2-ME. TCGF-I (fractions 79-81 from a) purified by an additional RP-HPLC step performed at a lower TFA concentration as described in the text. M_rs are shown $x10^{-3}$. * Units TCGF loaded in each lane. + RP-HPLC fraction number.

revealed only a single band with apparent Mr of 16,300 (Fig 3b) indicating that the Mr 32,000 band may be a disulfide-linked dimer of the biologically active Mr 16,300 protein. (The stained material at M_r 68,000 in figure 3b is an artifact, probably derived from 2-ME).

Table I summarizes the purification which was achieved. The total yield of TCGF biological activity was approximately 50% with purifications of 30,000 to 80,000 fold. In more recent experiments both TCGF-I and TCGF-II purified through the second RP-HPLC step stimulated [^{3}H]thymidine incorporation by TCGF-dependent human T cell lines (9) in a dose-dependent manner and were detectable at concentrations of less than 300 fg/ml (18 pM) and 100 fg/ml (6 pM), respectively (unpublished data).

Previous studies suggesting that TCGF may exist in multiple molecular forms have relied upon bioassay of partially purified material eluted from SDS-PAGE

gels (3,12). The results of such experiments are difficult to interpret quantitatively due to low and inconsistent recovery ($\leq$20% for the SDS-PAGE step alone) (12) of biological activity. It is thus not possible to evaluate the relative contributions of the separated activities to the total biological activity of the sample applied to the gel much less to the total biological activity present in the original starting material. It is also not clear that the substance responsible for the majority of the original biological activity has in fact been recovered and detected at all after electrophoresis. In addition the differences in mobility of the separated activities have not been confirmed upon re-electrophoresis. In contrast, we have resolved and purified to apparent homogeneity two biologically active species of TCGF. These species are routinely obtained in at least 50% overall yield from the starting conditioned medium and show consistent and reproducible differences in mobility upon rechromatography by RP-HPLC. TCGF-I and TCGF-II have apparent M_rs of 16,300 and 14,300 as judged by SDS-PAGE and protein staining of the purified preparations. These results provide strong evidence that these two molecularly distinct proteins are responsible for the majority of TCGF activity of MLA144 conditioned medium. Since no assumptions have been made regarding low recoveries of biological activity after exposure to denaturing conditions we may conclude that TCGF-I and TCGF-II have comparable specific activities. In addition, the ability to obtain homogeneous preparations of different molecular forms of TCGF in high yield will permit meaningful study of the structure and mechanism of action of this important lymphokine.

The structural basis for the difference in apparent molecular weight of TCGF-I and TCGF-II is unknown. This difference may result from variable sialation, as suggested for human TCGF (14), or incomplete proteolysis of the product of a single gene. Alternatively, TCGF-I and TCGF-II may represent the products of different structural genes. We are currently investigating these possibilities.

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