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ISOLATION AND PARTIAL CHARACTERIZATION OF HUMAN T-CELL GROWTH FACTOR

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Summary. Human T-cell growth factor (TCGF) has been isolated from conditioned media of the Jurkat T-leukemia cell line. Using a high-efficiency isolation procedure involving hollow fiber concentration, gel filtration and 3 steps of reverse-phase HPLC we obtained 100 to 600 pmol TCGF per liter of conditioned medium. Jurkat cell-derived TCGF (jTCGF) has a molecular weight of 15,750. The amino acid composition of jTCGF agrees well with that derived from the cDNA sequence coding for this protein (Taniguchi et al, Nature 302, 305, 1983). jTCGF is highly active in vitro in stimulating the proliferation of T-cells as measured by ^{3}H-thymidine incorporation into DNA (half-maximal stimulation with 3 fmol/100 µl well).

T-cell growth factor (TCGF), also termed Interleukin-2 (IL-2), has been recognized as an important member of the family of lymphokines which are lymphocyte-derived regulatory proteins that play a role in cellular and humoral immune responses. TCGF is released from antigen- or lectin-stimulated cultured T-cells or T-cell lymphomas and causes proliferation of activated T-lymphocytes (1) and of natural killer (NK) cells (2). It facilitates the continuous in vitro growth of functional T-cells (3) and NK cells (2), and if injected into tumor-bearing mice may cause an elevated immune response to the tumor (4).

Several laboratories have reported partial purification of human TCGF from normal lymphocytes (5-8) and the Jurkat T-leukemia cell line (6,7). Very recently, the production of homogeneous TCGF from the Jurkat cell line by immunoaffinity chromatography has been reported (9). Furthermore, the structure of a cDNA for human TCGF has been established by molecular cloning using mRNA from lectin-stimulated Jurkat cells (10). From these data the primary structure of jTCGF as a 133 amino acid single chain polypeptide

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comprising residues 21-153 of the precursor protein pre-TCGF has been proposed.

We report here the isolation of jTCGF using an approach based on the efficient combination of gel filtration and reverse-phase high-performance liquid chromatography (HPLC). Although the present procedure is not as elegant as the one devised by Robb et al (9) it should be useful as it does not rely on the availability of a monoclonal antibody to jTCGF.

MATERIALS & METHODS

TCGF production: The human Jurkat T-leukemia cell line was kindly provided by Dr. A. Altman (Scripps Clinic and Research Foundation, La Jolla, CA). It was cloned several times by limiting dilution in microtiter wells and a high producer clone (Jurkat E4) was isolated and expanded in RPMI 1640 containing 10% fetal calf serum. For induction of TCGF 10^6 cells/ml were cultured in RPMI 1640 supplemented with 10^{-5} M β-mercaptoethanol, 0.1 mM non-essential amino acids, 1mM L-glutamine and 1 mM pyruvate. Lymphokine production was induced by addition of 10 μg/ml of phytohemagglutinin (Gibco) and 10 ng/ml of phorbol 12-myristate 13-acetate (Sigma) at the time of plating. Conditioned medium was collected after 36-48 h by centrifugation and filtered through a 0.45 μm Millipore membrane.

Bioassay: For assay of TCGF the mouse cell line CTLL-2 (kindly provided by Dr. M. Thoman, Scripps Clinic and Research Foundation) was used. Cells were maintained in culture in RPMI 1640 medium supplemented with 30% supernatant derived from Con A stimulated rat splenocytes (2). Exponentially growing CTLL-2 cells were cultured for 24 h prior to their use in the bioassay in RPMI 1640 medium (without Con A supernatant) at a concentration of 10^5 cells/ml. For assay purposes cells were adjusted to a concentration of 6×10^4 cells/ml. Fifty μl of cell suspension were mixed with 50 μl of test sample in complete RPMI 1640 medium, cultured in triplicate and pulsed on day 2 with 0.5 μCi ^{3}H-thymidine/well for 5 h. Cells were collected with an automatic harvester and radioactivity determined by liquid scintillation counting. One TCGF unit was defined as the amount causing half maximal incorporation of ^{3}H-thymidine into DNA under the assay conditions described.

Isolation: Batches of 300-2000 ml conditioned medium were concentrated to a volume of 60 ml on a hollow fiber device (Amicon, Model CH-4, fiber cartridge no. H1P5-43) at 4°C. Concentrated media were subjected to gel filtration on Sephacryl S-200. Reverse-phase HPLC was performed on microprocessor-controlled gradient HPLC equipment (Altex) using Model 110A pumps and the following chromatographic systems: (a) a semipreparative C8 column (Altex Ultrasphere, 25 x 1 cm, 5 μm particle and 100 A pore size) with 1 M pyridine acetate, pH 4/n-propanol as mobile phase; (b) ag analytical C3 column (Altex RPSC, 7.5 x 0.46 cm, 5 μm particle size, 300 A pore size) with 0.1% (v/v) trifluoroacetic acid/acetonitrile as mobile phase; and (c) an analytical C4 column (Vydac, 25 x 0.46 cm, 5 μm particles, 330 A pore size, Separations Group, Hesperia CA) with the same mobile phase as in (b). Column effluents were monitored for protein content by measuring UV light absorbance at 210 or 280 nm with a Schoeffel-Kratos variable wavelength UV detector or by fluorescence detection using the fluorescamine stream-sampling detector (11). For the determination of specific activities protein content was estimated by amino acid analysis.

Structural characterization. Amino acid analysis: Amino acid analyses of hydrolysates of 5-10 pmol protein were performed with a Liquimat III amino acid analyzer (Kontron, Zurich, Switzerland) as described (12). Amino acid

sequence analysis by automated Edman degradation of purified protein was performed with an Applied Biosystems Model 470A gas-phase sequencer (13). Phenylthiohydantoin amino acid derivatives were identified by reverse-phase HPLC (14). The molecular weight of jTCGF was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis according to the procedure of Laemmli (15) using a 15% polyacrylamide gel. Gel bands were visualized by silver staining as described (Biorad kit and procedure). Lectin chromatography: pmole quantities of TCGFs were chromatographed on a 1 ml Con-A Sepharose-4B column (Pharmacia) with α-methyl mannoside as the displacing ligand, as described previously (16).

RESULTS AND DISCUSSION

A typical isolation of jTCGF is illustrated in Fig. 1. The final product was judged highly pure by means of sodium dodecyl sulfate polyacrylamide gel electrophoresis which revealed a single silver-stained band (data not shown) and by amino acid analysis (see below). Polyacrylamide gel electrophoresis indicated a molecular weight of 15,750 for jTCGF (data not shown) which is in excellent agreement with that of a 133 amino acid protein predicted from the jTCGF cDNA sequence. Overall recovery of jTCGF was excellent (>80%). In 2 separate isolations starting with 350 and 2000 ml of conditioned medium, approximately 200 and 140 pmol of jTCGF was obtained. It is conceivable that this variation is due to differences in growth factor content of the conditioned media, possibly because of variations in the effectiveness of growth factor induction by lectin and phorbolester.

jTCGF causes cell proliferation as determined by thymidine incorporation into DNA of cultured T-lymphocytes *in vitro* (Fig. 2) in a dose-dependent manner. Half maximal stimulation of DNA synthesis occurs at a growth factor concentration of 30 pM and the minimal detectable concentration is approximately 1 pM.

The amino acid composition of (Table 1) agrees well with that of the amino acid sequence 21-153 of pre-TCGF derived by molecular cloning of Jurkat mRNA (10). Microsequence analysis of 160 pmol jTCGF revealed the amino terminal residue alanine, which is in agreement with that of Robb et al (9), who established that the amino acid sequence of jTCGF begins with alanine at position 21 of the precursor protein. The initial low yield (10%) prevented the sequential assignment of further amino acids.

Previous evidence indicates that jTCGF displays molecular heterogeneity (9, 17), probably due to molecules of varying carbohydrate content. The present data confirm this observation: quantitatively minor forms of TCGF are separated from the major TCGF species by reverse phase HPLC (Fig 1).

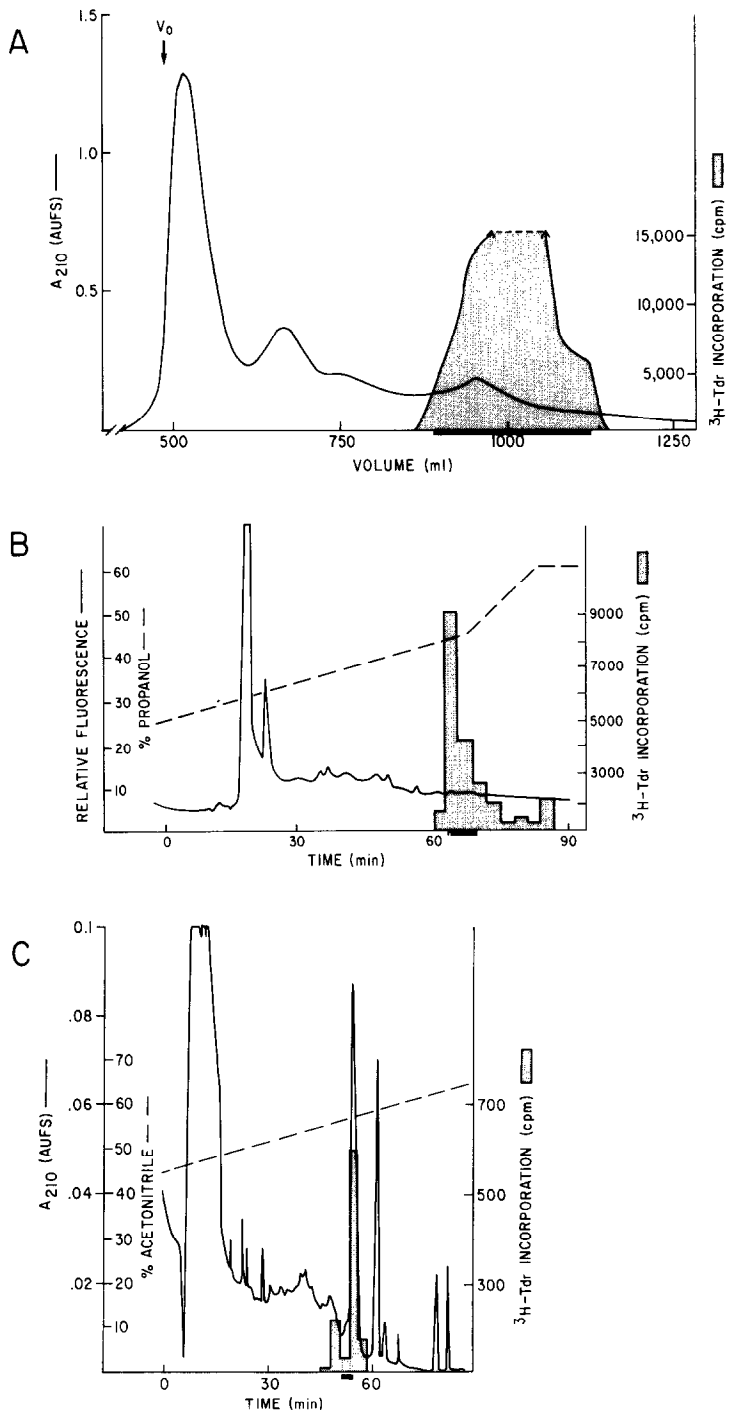

Figure 1:

A: Gel filtration of concentrated conditioned medium on Sephacryl S-200 (95 x 5 cm). Sample: 60 ml hollow fiber concentrate (corresponding to 2000 ml conditioned medium). Elution buffer: 10 mM Tris Cl, pH 7.5/0.5 M NaCl; flow rate: 66 ml/h; temperature: 4°C. Fractions of 11 ml were collected and aliquots of 10 µl or less used directly for bioassay. Active fractions were stored at

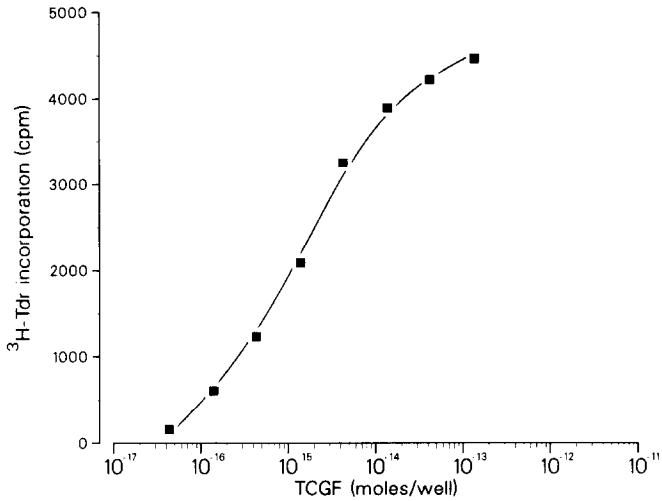

Figure 2: Dose response curve of jTCGF with HPLC-purified material (after preparation of aliquots for bioassay as described in Fig 1B). Individual points are averages of triplicate determinations with standard deviations being less than 7%. Wells contained cells suspended in 100 µl of medium.

Because of the possibility that jTCGF may contain carbohydrate (7,9), highly purified jTCGF was subjected to chromatography on Con A-Sepharose. jTCGF did not bind to the affinity gel, suggesting that it does not possess carbohydrate moieties recognizable by Concanavalin A. This result confirms earlier data by Mier & Gallo (5), who did not find any binding of TCGF to several types of lectins. However, Robb et al (9) have now determined that a major form of jTCGF contains N-acetyl-D-galactosamine attached to the threonine residue in position 3 of the polypeptide chain.

Fig. 1. Continued.

4°C until further use. V_0 = exclusion volume of the column. The horizontal bar indicates active fractions pooled for further purification.

B: Semi-preparative reverse-phase HPLC of gel filtration fractions on a C8 column using pyridine acetate, pH 4/n-propanol as eluant. Flow rate: 0.8 ml/min. For loading, the pool of bioactive fractions (300 ml) from gel filtration (Fig 1B) was pumped directly through the column prior to starting the propanol gradient for TCGF elution. Fraction size: 3.2 ml. Aliquots of 16 µl of each fraction were added to 100 µg/human serum albumin (1 mg/ml), dried in a Speed-Vac (Savant) vacuum centrifuge and subjected to bioassay. The horizontal bar indicates fractions pooled, diluted 3-fold with 0.2 M acetic acid, loaded onto an analytical C3 column and eluted with 0.1% (v/v) trifluoroacetic acid in an acetonitrile gradient (data not shown).

C: Analytical reverse-phase HPLC of bioactive fractions from the C3 column (see legend to Fig. 1B) on a C4 column using the trifluoroacetic acid/acetonitrile mobile phase. Flow rate: 0.6 ml/min fraction size: 1.8 ml. Bioassay was performed as described under Fig. 1B.

TABLE 1: AMINO ACID COMPOSITION OF TCGF[a]

	Jurkat TCGF	cDNA[b]
Asx	11.43 ± 0.56	12
Thr	11.78 ± 1.37	13
Ser	9.15 ± 1.20	8
Glx	17.93 ± 1.02	18
Pro	8.05 ± 0.64	5
Gly	2.95 ± 1.46	2
Ala	4.88 ± 1.05	5
Cys[c]	3.90 ± 0.14	3
Val	2.88 ± 0.10	4
Met	4.08 ± 0.33	4
Ile	6.58 ± 0.34	9
Leu	22.73 ± 1.11	22
Tyr	3.28 ± 0.40	3
Phe	5.93 ± 0.57	6
His	2.00 ± 0.22	3
Lys	11.83 ± 3.56	11
Trp	0.37 ± 0.06	1
Arg	5.88 ± 0.78	4

a Amino acid compositions are derived from 24 h hydrolyzates using 5-10 pmol of protein. Values are not corrected for hydrolysis-induced losses and are means ± standard deviations (4 determinations except for Pro and Cys analyzed in duplicate).
b Theoretical amino acid composition derived from cDNA sequence (10).
c Determined as cysteic acid.

The significance of our study lies in the fact that we have designed an efficient procedure for the isolation of human TCGF. It is noteworthy that the isolation of TCGF was achieved by relying almost entirely on reverse-phase HPLC. In this context we emphasize the use of the relatively new C3 and C4 reverse-phase columns. TCGF is a rather hydrophobic protein and therefore may be easily lost by adsorption to surfaces due to hydrophobic interaction. Indeed, previous attempts at purifying TCGF have been hampered by such loss (6). In our own work we have noted losses and poor chromatographic behavior (broad peaks) when the highly hydrophobic C18 stationary phase or even C8 columns (Fig. 1B) were used. The less hydrophobic C3 and C4 reverse phases, however, provided good recovery and high resolution and are considered the columns of choice for protein separation problems. The efficiency of the proposed isolation procedure is illustrated by the fact that microgram amounts of TCGF can be isolated from relatively small quantities of conditioned medium.

The purification method described here is simpler than another HPLC-based procedure devised by Milstone & Porter who purified two forms of Gibbon TCGF

using a C18 reverse-phase column in the final steps (18). The present procedure is not as simple and efficient as the one recently described by Robb et al (9) who used a monoclonal antibody against jTCGF to purify the growth factor from conditioned medium with a one-step immunoaffinity chromatography procedure. However, the present method does not rely upon a specific antibody and therefore should be useful in situations where such an antibody is not available. Furthermore, this method should allow for the purification of other major forms of TCGF, particularly those present in normal blood lymphocytes that do not interact with the monoclonal antibody generated by Robb et al (9).

The potential applications of TCGF in experimental biology and clinical medicine are plentiful. The availability of pure TCGF will lead to the production of specific antibodies suitable for the development of radioimmunoassays for experimental purposes and for the diagnosis of diseases of the immune system. The in vitro propagation and cloning of various types of T-cells and natural killer cells using TCGF should be important for the understanding of the precise biological functions of these cells. Finally, TCGF may have therapeutic potential for patients whose cellular immune system is impaired.

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