Proc. Natl. Acad. Sci. USA
Vol. 77, No. 10, pp. 6134–6138, October 1980
Medical Sciences

Purification and some characteristics of human T-cell growth factor from phytohemagglutinin-stimulated lymphocyte-conditioned media

(lymphokines/blast transformation/cultured T cells/colony-stimulating activity)

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Communicated by Jack Leonard Strominger, June 16, 1980

ABSTRACT **Human T-cell growth factor (TCGF), a mitogenic protein that appears in the media of cultured lymphocytes after phytohemagglutinin-stimulation, has been purified more than 400-fold from serum-free conditioned media by using a sequence of ion exchange chromatography and gel filtration. The purified growth factor elutes as a broad peak from DEAE-Sepharose, focuses diffusely at a pH of about 6.8 on isoelectric focusing (suggesting heterogeneity in electrical charge), has an estimated molecular weight of approximately 23,000 as judged by gel filtration (12,000–13,000 on Na-DodSO$_4$/polyacrylamide gel electrophoresis), is resistant to DNase and RNase, is degraded by trypsin, and does not adhere to any of several lectin-Sepharoses. These characteristics indicate that it is nonglycosylated and protein in nature. The activity of the factor, determined by cell counts or [^{3}H]thymidine incorporation in human T lymphoblasts, is stable at room temperature in crude conditioned media, but the partially purified factor requires the addition of albumin or polyethylene glycol to maintain stability. Unlike the crude conditioned media, the purified factor lacks colony-stimulating activity and, unlike lectins, antigens, and crude conditioned media, it does not initiate blastogenesis in peripheral blood lymphocytes but is a selective mitogen for T cells that have undergone blast transformation secondary to exposure to a lectin or antigen. This indicates that the factor is a second signal in the T-cell immune response. The partially purified factor has been used to selectively grow several human T-cell lines, including cells that are cytotoxic to a variety of target cells.**

The discovery of a factor termed T-cell growth factor (TCGF) in phytohemagglutinin (PHA)-stimulated lymphocyte-conditioned media (Ly-CM) that allowed the long-term propagation in suspension culture of human T lymphocytes from peripheral blood or bone marrow has been reported previously (1, 2). These cells have been maintained in culture for periods of more than 1 year and have retained their dependency on the growth factor. They have been shown to be negative for Epstein–Barr nuclear antigen, to lack surface immunoglobulin, and to have the rosetting characteristics of T lymphocytes. Furthermore, even after months in culture, they have retained a normal karyotype and do not produce tumors in *nude* mice. Others have confirmed these findings and extended them to the murine and rat systems (3–5). In some laboratories, concentrates of the crude Ly-CM have been used to generate cytotoxic T cells (6–9) and to investigate the role of cultured T cells in helper/suppressor assays (10).

The coexistence of colony-stimulating factors, interferon, and T-cell replacing factors (11), as well as of various mitogenic substances such as PHA, lymphocyte-activating factors (12, 13), and TCGF in the crude Ly-CM has made it difficult to assign a definite biological function to any one particular protein independent of the others. Therefore, our first objective was to purify TCGF from human Ly-CM and to determine its biochemical and biological characteristics. Recently, Kurnick *et al.*, working with lectin-free crude Ly-CM, were able to differentiate the biological effects of TCGF, which stimulates only T lymphoblasts, from those of PHA, which induces proliferation in peripheral blood lymphocytes (5). However, to date, no one has purified to homogeneity any of the factors present in the crude Ly-CM. In this paper, we report an extensive purification of human TCGF from serum-free Ly-CM and describe some of its characteristics.

EXPERIMENTAL PROCEDURES

Materials. DEAE-Sepharose was obtained from Pharmacia and Ultrogel AcA54 was obtained from LKB. The UM-05 membrane and ultrafiltration cell used to concentrate the protein samples were purchased from Amicon (Lexington, MA). The Millex 0.22-μm filters used to sterilize samples for biological assay were obtained from Millipore. [^{3}H]Thymidine was purchased from Schwarz/Mann. Whatman 2.4-cm GFC glass fiber filters were used in the biological assays. Lectin-Sepharoses were purchased from P-L Biochemicals. Acrylamide for gel electrophoresis was obtained from Fisher.

Assay for TCGF. PHA-stimulated lymphocytes were prepared from the heparinized blood of healthy laboratory personnel. Lymphocyte separation medium (Pharmacia) was used to obtain the mononuclear cells, which were incubated at 10^6 per ml in RPMI 1640 (GIBCO) containing 20% fetal calf serum and PHA-*P* (10 μg/ml) at 37°C in a 5% CO_2/95% air humidified incubator. Every 5–7 days the cells were split and maintained by an appropriate dilution of an $(NH_4)_2SO_4$ precipitate of the Ly-CM as described (1). At this stage, cultured T blasts are not appreciably stimulated by PHA and, therefore, these cells were the target cells for all subsequent TCGF assays. Samples (0.2 ml) of the various fractions to be assayed were added to a lymphocyte suspension of approximately 2×10^5 cells, and RPMI containing 20% fetal calf serum was added to a total volume of 2.0 ml. All materials assayed were sterile and in physiologic buffers. The cell suspensions were incubated in 15-ml conical centrifuge tubes at 37°C for 48 hr, at which time 0.2 ml of [^{3}H]thymidine (10 mCi/ml. 0.36 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was added. The thymidine incorporation was determined 18 hr later. The cell pellets were repeatedly washed in RPMI lacking fetal calf serum, and 0.15 ml of 90% Cl_3CCOOH was added to the 2.0-ml suspension. The precipitates were collected on glass fiber filters and rinsed with 5% Cl_3CCOOH and 70% EtOH. Liquid scintillation counting of the dried filters was performed with 7 ml of Liquifluor solution [42 ml of Liquifluor (New England Nuclear) per liter of tolu-

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Abbreviations: PHA, phytohemagglutinin; TCGF, T-cell growth factor; Ly-CM, lymphocyte-conditioned media; PEG, polyethylene glycol; P_i/NaCl, phosphate-buffered saline.

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ene]. Serial dilutions of pooled active fractions from the various purification steps were assayed as described (3) to quantitate the TCGF activity and calculate the percentage yields. Protein determinations for the specific activity computations were performed by using the Lowry method (14). The fractions obtained with the various chromatographic methods used in the purification sequence have been independently and repeatedly assayed for total number of T lymphocytes as the criterion for T-cell growth; in every instance, there was complete agreement between this assay and the more rapid thymidine incorporation technique.

Assay for Colony-Stimulating Factor. Colony-stimulating factor was assayed in the soft gel system by using methylcellulose, as described (15). Briefly, each milliliter of culture contained 0.8% methylcellulose, 15% preselected fetal calf serum, 2×10^5 human bone marrow cells, and 10% test sample. A conditioned medium from human placenta (16) was used as a positive control source of this factor (a gift of A. Burgess, Melbourne, Australia). Cultures were incubated at 37°C in a fully humidified atmosphere of 10% CO_2/90% air. Colony size (>50 cells) and number were scored on days 7 and 14.

STEPS IN THE PURIFICATION OF HUMAN TCGF

Crude Ly-CM. Human mononuclear cells obtained from the peripheral blood of multiple donors were incubated at 10^6 cells per ml in RPMI 1640 in the absence of serum but the presence of 0.25% bovine serum albumin and PHA-*P* at 10 μg/ml at 37°C for 72 hr. The cells were then removed by centrifugation, and the medium was frozen until beginning the purification of TCGF.

Filtration and Ammonium Sulfate Precipitation. Approximately 1500 ml of crude Ly-CM was first filtered through 0.45-μm Nalgene filters to remove gross debris. Sufficient $(NH_4)_2SO_4$ to produce a 50% saturated solution was gently added and, after stirring for 1 hr at 4°C, the precipitate was removed by centrifugation at 10,000 × *g* for 10 min. $(NH_4)_2SO_4$ was then added to the supernatant to produce 75% saturation; after stirring for 1 hr, the suspension was centrifuged as above. The precipitate was dissolved in approximately 70 ml of 0.01 M Tris·HCl (pH 8.0), and sufficient phenylmethylsulfonyl fluoride was added to yield a 0.1 mM solution. The dissolved precipitate was then dialyzed against 50 vol of the above Tris·HCl buffer for 48 hr with at least one change of dialysate.

Anion Exchange Chromatography. All chromatographic procedures were performed at 4°C. The dialyzed concentrate was placed on a 35-ml column of DEAE-Sepharose previously equilibrated with the Tris buffer. After the sample had entered the gel, the column was rinsed with 35 ml of buffer, and a 250-ml linear gradient of Tris-buffered NaCl terminating at 0.2 M NaCl was applied. A flow rate of 15 ml/hr was maintained, and 7-ml fractions were collected. TCGF activity eluted as a broad peak or a minimally resolved doublet (Fig. 1) that centered at approximately 0.07 M NaCl. The active fractions (nos. 23–29) were pooled, and 50 ml of 10% polyethylene glycol (PEG) 6000 was added. The resulting solution was reduced to 5 ml by using a Diaflo UM-05 membrane.

Gel Filtration. The preparation obtained after ion exchange chromatography was applied to a column of Ultrogel AcA54 (2.5 × 90 cm) and eluted with phosphate-buffered saline Pi/NaCl/0.1% PEG at a flow rate of 15 ml/hr (Fig. 2). Five-ml fractions were collected. TCGF activity was localized in fractions 63–73, which were pooled. The center of this region of the elution profile corresponds to a molecular weight of approximately 23,000 (17). This chromatographic step is an important

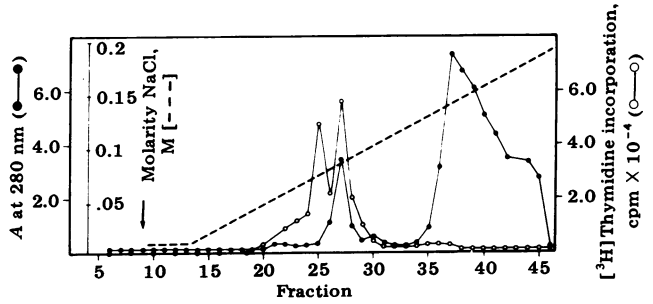

FIG. 1. DEAE-Sepharose elution profile. Proteins were eluted with a linear Tris-buffered NaCl gradient of 0–0.2 M. TCGF activity was determined by the [³H]thymidine incorporation assay. The arrow denotes the beginning of the NaCl gradient. Fractions 23–29 were pooled for further purification.

one that eliminates approximately 97% of the remaining protein. Recovery of biological activity through the $(NH_4)_2SO_4$ precipitation, ion exchange chromatography, and Ultrogel filtration steps has routinely been in the 10% range as determined by assaying serial dilutions of pooled active fractions at different stages in the purification. The specific activity at this point in the purification sequence is approximately 400-fold greater than that of the crude Ly-CM. The mitogenic effect of this material on cultured T cells is shown in Fig. 3.

Isoelectric Focusing. The concentrate after gel filtration on Ultrogel was dialyzed against 0.01 M Tris·HCl (pH 8.0), and 0.5 ml of glycerol and 0.1 ml of Ampholines (pH range 3.5–10.0; LKB) were added. A 5–60% glycerol density gradient, including 0.1% PEG and 2% Ampholines, was layered into a preparative isoelectric focusing column (ISCO, model 212), and the protein sample was injected into the isodense region of the gradient. The focusing was carried out at 1000 V for 48 hr at 4°C. Fractions (1 ml) were collected, and their pHs were determined. The glycerol and Ampholines were removed by dialysis for 24 hr against 100 vol of P_i/NaCl and the TCGF activity of the various fractions was assayed. The TCGF was focused diffusely about a pH of 6.8, suggesting some degree of electrical heterogeneity (Fig. 4). The recovery of TCGF activity after isoelectric focusing has been too low to favorably affect the specific activity; thus, this method is not routinely used as a purification step. Nonetheless, it provides an excellent means of separating TCGF from residual colony-stimulating activity which focuses toward the anode.

Preparative NaDodSO₄/Polyacrylamide Gel Electrophoresis. A 280 × 1.5 mm 13% polyacrylamide slab gel having a 3% spacer was prepared by using a Bio-Rad model 221 gel apparatus. The acrylamide solutions, containing riboflavin (5

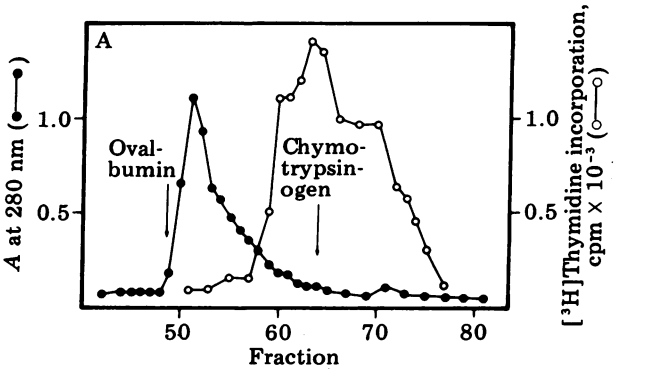

FIG. 2. Gel filtration on Ultrogel AcA54. TCGF activity was determined by [³H]thymidine incorporation assay. Arrows denote the elution points of ovalbumin (Ova) and chymotrypsinogen (Chg). Fractions 63–73 were pooled for further purification.

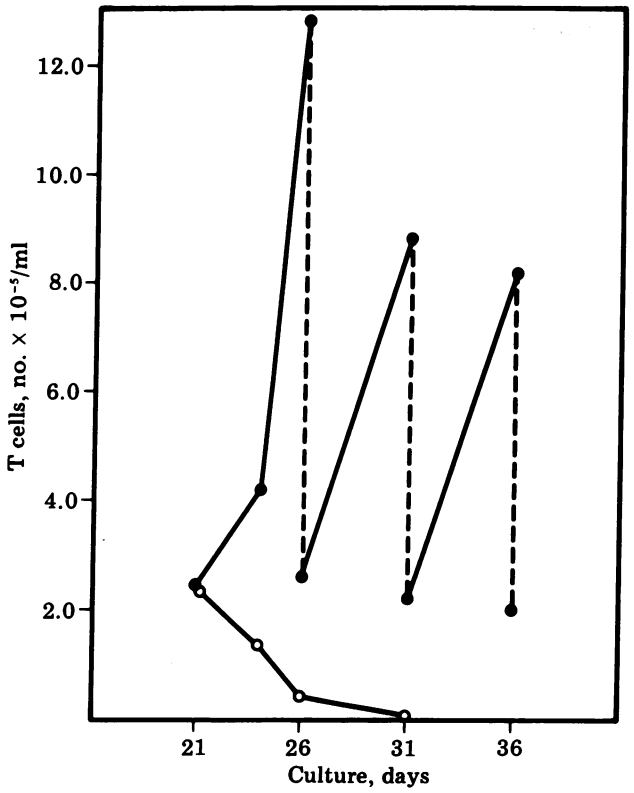

FIG. 3. Growth of human T cells in presence of TCGF purified by using DEAE-Sepharose and Ultrogel AcA54 chromatography. Growth of cultured T cells in medium containing (●) and lacking (○) TCGF. The dashed line refers to culture splits done at 5-day intervals.

mg/liter), were polymerized by exposure to a fluorescent light source for aproximately 1 hr, as described (18). The gel mixture and reservoir buffer contained 0.1% $NaDodSO_4$.

The Ultrogel fractions containing TCGF were dialyzed against the Tris·HCl buffer and concentrated to 2 ml by stepwise elution from a DEAE-Sepharose column using 0.5 M NaCl/10 mM Na_2HPO_4, pH 7.4, as the eluting buffer. In preparation for gel electrophoresis, the concentrate was again dialyzed against Tris·HCl and then denatured by treatment with 1% $NaDodSO_4$ (without 2-mercaptoethanol) at 37°C for 1 hr. Glycerol and bromophenol blue were added. The TCGF sample was then electrophoresed at 60 V until the dye had condensed to a thin line in the running gel, at which time the voltage was increased to 120. Sixteen hours later, the gel was sliced into 0.5-cm-thick horizontal slices after removal of a center longitudinal strip for staining. The slices were macerated by passage through a 3-ml syringe, and 5 ml of P_i/NaCl/0.1% PEG was added to each fraction. The TCGF was extracted by gentle agitation overnight at room temperature, and the $NaDodSO_4$ was removed by passage of the solution over 0.5-ml Dowex 1 × 2 columns (Bio-Rad). The fractions were dialyzed against P_i/NaCl, passed through Millipore filters, and assayed. Several unsuccessful attempts to detect TCGF activity in the upper two-thirds of the gel have obviated the need for routine assaying of this region. Protein bands were made visible in the longitudinal strip by using a modification of the silver staining technique (19).

The results of the assays were correlated with specific stained protein bands (Fig. 5). The peak of TCGF activity, fraction 35, was associated with a pair of juxtaposed bands that migrated in the low-molecular-weight region (12,000–13,000) in an analytical $NaDodSO_4$ gel.

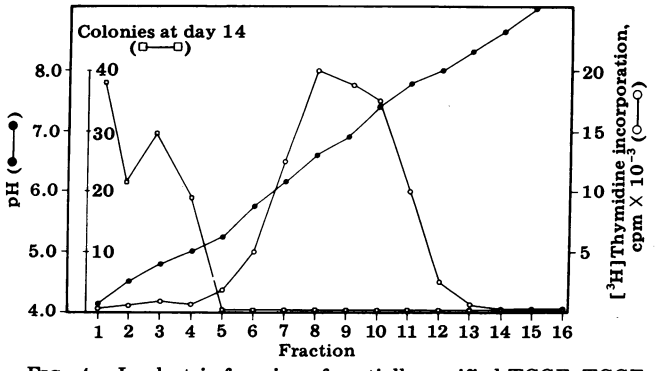

FIG. 4. Isoelectric focusing of partially purified TCGF. TCGF activity was determined by [^{3}H]thymidine incorporation assay. TCGF was focused as a diffuse peak centered at pH 6.8. Colony-stimulating (□) activity was assayed with normal human marrow.

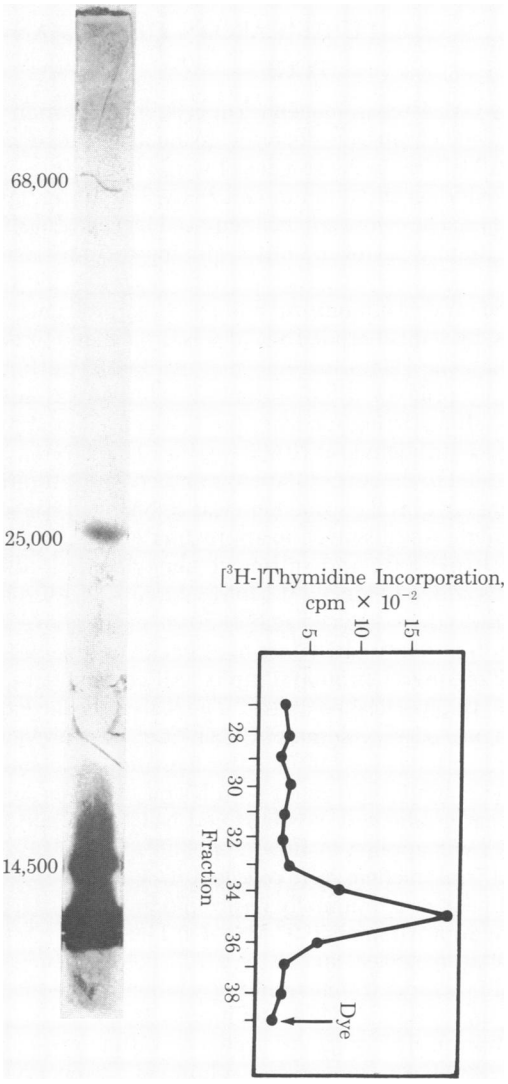

FIG. 5. Preparative polyacrylamide gel electrophoresis of partially purified TCGF. TCGF extracted from the sliced polyacrylamide gel was assayed, and the activity profile (●—●) is shown aligned with the stained gel. The active fraction was associated with a pair of juxtaposed bands of molecular weight 12,000–13,000. TCGF activity has never been recovered from the upper two-thirds of the gel, which is therefore not routinely assayed.

RESULTS

Experiments with Sepharose-Immobilized Lectins. Several immobilized lectins capable of binding glycoproteins having specificities determined by the terminal sugar were used in attempts to further purify TCGF. TCGF purified by $(NH_4)_2SO_4$ precipitation and Sephadex G-100 chromatography was dialyzed against a 0.2 M NaCl/0.01 M K_2HPO_4, pH 6.5/1 mM $CaCl_2$/1 mM $MgCl_2$/0.2 mM MnCl. Samples (2 ml) of this material were placed on 1-ml columns of Sepharose-bound concanavalin A and lentil lectin; after the columns were rinsed with buffer until the absorbance had reverted to baseline, 0.1 M methylmannose in buffer was applied. The nonadherent protein fractions and those eluted with the competing sugar were dialyzed against P_i/NaCl, and the TCGF activity was determined. In both cases, the TCGF was found solely in the nonadherent fractions. Similar experiments were done by using PHA-Sepharose and peanut lectin-Sepharose, the same buffer, and *N*-acetylgalactosamine as the competing sugar (Fig. 6). TCGF was also applied to a column of fetuin-Sepharose; after the column was rinsed, a mixture of 0.5 M NaCl/0.05 M glycine-HCl, pH 3.0, was applied. In each instance, TCGF was recovered exclusively in the nonadherent fractions, suggesting that human TCGF is not glycosylated.

Specificity of TCGF for T Lymphoblasts. Minimally purified TCGF was able to discriminate between unactivated human peripheral blood lymphocytes and T lymphoblasts that had been obtained by activation with PHA (Fig. 7). The [^{3}H]-thymidine incorporation induced by the unprocessed Ly-CM in peripheral blood lymphocytes is consistent with residual PHA in the crude media. Although the incorporation of [^{3}H]thymidine by fresh lymphocytes treated with purified TCGF was higher than that of the unstimulated negative controls, it was only negligibly so. Conversely, lymphoblasts obtained after exposure to PHA were quite sensitive to the purified TCGF and showed a meager response to the lectin. Human TCGF is capable of sustaining the long-term growth in suspension culture of T lymphoblasts from several animal sources, including humans, gibbon apes, rhesus monkeys, rats, and mice (unpublished data).

Miscellaneous Biochemical Characteristics of Purified Human TCGF. Efforts to alter the biological activity of partially purified TCGF by exposure to degradative enzymes, reducing agents, and electrophilic organic reagents have provided additional information regarding the chemical structure of human TCGF. Treatment with DNase or RNase had a negligible effect on the potency of the factor, whereas trypsin abolished all activity, showing that TCGF is a protein. Treatment with the serine-specific protease inhibitor phenylmethylsulfonyl fluoride or with the sulfhydryl alkylator *N*-ethylmaleimide had no effect on the biological activity, suggesting that the mechanism of action of TCGF is not proteolytic and does not involve a strategically located cysteine. Exposure to 1 mM dithiothreitol or 2-mercaptoethanol likewise had no effect. The lectin-Sepharose experiments indicate that human TCGF either lacks a polysaccharide component or that, if initially present, the carbohydrate is lost in a degradative process and is not a determinant of biological activity.

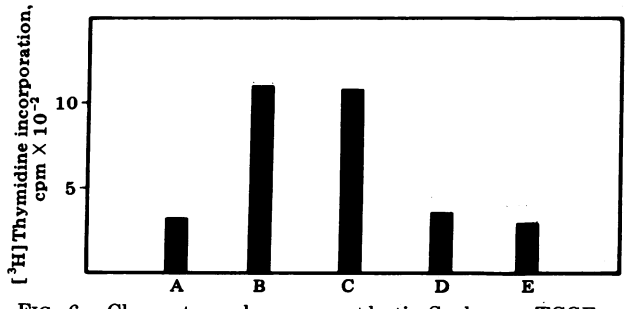

FIG. 6. Chromatography on peanut lectin-Sepharose. TCGF was applied to a 1.0-ml column of peanut lectin-Sepharose. Essentially all of the TCGF activity was found in the nonadherent fractions and none in the sugar or sugar/ethylene glycol fractions. A, Control; B, TCGF sample; C, nonadherent fractions; D, fraction eluted with *N*-acetylgalactosamine; E, fraction eluted with *N*-acetylgalactosamine in 50% ethylene glycol.

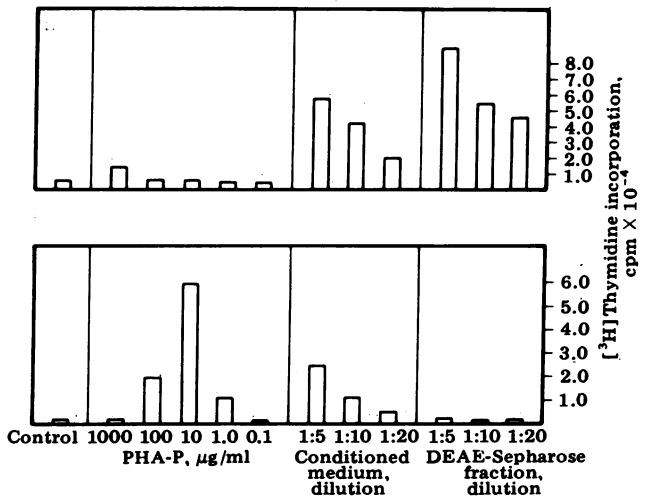

FIG. 7. Comparison of effects of PHA and partially purified TCGF on fresh human peripheral blood lymphocytes (*Lower*) and on T lymphoblasts (*Upper*). T lymphoblasts were cultured for 8 days after exposure to PHA. The DEAE-Sepharose fractions preferentially stimulated the lymphoblast cells; PHA, however, initiated lymphoblast transformation from fresh peripheral blood lymphocytes, but showed no significant effect on the T lymphoblast cells.

DISCUSSION

We chose to prepare the crude Ly-CM from lymphocytes incubated in a serum-free medium containing 0.25% bovine serum albumin. The deletion of the serum markedly simplified the purification, and the chemical properties of bovine serum albumin allowed a quantitative removal in the ion exchange step. In our experience, this preparation represents an ideal compromise between purity and potency, because TCGF obtained after DEAE-Sepharose chromatography has a much higher specific activity than the crude Ly-CM and a marked specificity for T lymphoblasts (Table 1).

One of the major obstacles in the purification of TCGF was its instability at low protein concentrations. Despite the stability of TCGF activity in the crude Ly-CM, preparations processed through the ion exchange and molecular sieve steps rarely survived more than 1 week without specific additives. Deter-

Table 1. Purification of human T-cell growth factor

Fraction	Protein, mg	Total activity, cpm	Yield, %	Specific activity, cpm	Purification, fold
Crude Ly-CM	4396	1570	100	0.36	1.0
$(NH_4)_2SO_4$ ppt	1500	1050	67	0.70	1.94
DEAE-Sepharose	30	450	28	15.0	41.6
Ultrogel	0.9	138	9	153	425

One unit of TCGF activity was arbitrarily defined as the amount of TCGF in 1 ml of crude Ly-CM. Total activity of the different fractions was computed by multiplying the volume of the fraction by its potency relative to the crude material, as determined by assaying serial dilutions.

gents such as Triton X-100 had no protective effect over a wide range of concentrations. Freezing at −70°C or addition of reducing agents such as 2-mercaptoethanol or of the protease inhibitor phenylmethylsulfonyl fluoride, both of which were removed by dialysis before the bioassay, likewise failed to prevent loss of activity. The addition of 0.25% bovine serum albumin or 0.1% PEG 6000 stabilized the purified material for periods of several months, whereas small carbohydrates such as ethylene glycol and glycerol had no discernible effect. The mechanism by which PEG stabilized TCGF is not known. Because PEG has been reported to augment lymphocyte proliferative responses in mixed lymphocyte reactions (20), it was important to differentiate a protective effect of PEG on TCGF from a direct stimulatory effect of PEG on T cells. Solutions containing 0.1% PEG do not induce levels of [^{3}H]thymidine incorporation above background in our assays. Furthermore, the concentrations of PEG and other hydrophilic polymers that have been shown to enhance lymphocyte proliferative responses in mixed lymphocyte reactions are 50-fold greater than those used here. We suspect that the apparent protective effect of PEG on purified TCGF is due to the prevention of undesired hydrophobic interaction between TCGF and container surfaces.

Kurnick *et al.* have shown that the removal of PHA by using an affinity column did not adversely affect the potency of the Ly-CM in assays using T lymphoblasts as the proliferating cells (5). In contrast to unstimulated, fresh peripheral blood lymphocytes, T lymphocytes that have previously undergone lymphoblast transformation due to previous treatment with a lectin or exposure to an antigen proliferate in response to PHA-free Ly-CM. We observed a similar phenomenon early in the TCGF purification sequence in that fractions from the DEAE-Sepharose step have a comparable degree of selectivity in stimulating T lymphoblasts but not peripheral blood lymphocytes. It is evident that an initial activating step is a requisite for the proliferative response to TCGF. The specificity of the growth factor for lymphoblasts, noted in Fig. 7, is consistent with studies carried out with crude material in which T lymphoblasts, but not peripheral blood lymphocytes were shown to absorb the TCGF from Ly-CM (21). TCGF is apparently a second signal in the response of T cells to an antigen; a corollary of this is that the activation of T lymphocytes to form T lymphoblast cells involves the appearance of a specific receptor for TCGF at the level of the plasma membrane.

Jurjus *et al.* have demonstrated that partially purified TCGF obtained by DEAE-Sepharose chromatography can be used to grow cytotoxic T cells in suspension culture from patients who have acute myelogenous leukemia (22). Unlike the polyclonally activated T cells obtained by lectin stimulation, the cells grown with partially purified TCGF show some specificity in killing autologous leukemic myeloblasts and do not have appreciable cytotoxicity against other target cells, except for crossreactivity for other human leukemic myeloblasts. This observation is consistent with the proposed model of TCGF action, in that purified TCGF selects for T cells that have been activated by previous antigen exposure.

Preparative gel electrophoresis has clearly identified the TCGF activity with a pair of protein bands of molecular weight 12,000–13,000. Although the active fraction after electrophoresis was quantitatively assayed by serial dilution and found to be approximately 4 times as potent as the crude Ly-CM (data not shown), the yield was low. TCGF eluted from the gel, however, was capable of inducing T-cell proliferation. It is unclear why the molecular weight of TCGF in a dissociating gel deviates to such a large extent from that predicted by gel filtration; however, several explanations are tenable. The apparently high molecular weight in Ultrogel chromatography may be the result of aggregation or complexing with contaminant proteins; if this is the case, the question of which of the two protein bands is the active one is unanswered. The widths of the activity profiles in isoelectric focusing, ion exchange chromatography, and gel filtration are consistent with heterogeneity and, therefore, both bands may be active. Another possibility is that the two bands in fraction 35 represent subunits of the native TCGF molecule (the molecular weights are consistent with this).

Although the literature on TCGF is expanding rapidly, in most experiments done thus far crude preparations containing PHA, colony-stimulating factor, interferon, and numerous other factors have been used. The purification sequence discussed here gives a TCGF preparation that totally lacks colony-stimulating factor and is incapable of stimulating peripheral blood lymphocytes.

We thank Mr. Steve Kishter for excellent technical assistance and Ms. Andrea Woods for performing the colony-stimulating factor assays.

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