

PURIFICATION OF MURINE T CELL GROWTH FACTOR

A Lymphocyte Mitogen with Helper Activity*

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The regulation of *in vitro* immune functions (lymphocyte proliferation, antibody production, macrophage activity, cytotoxicity, etc.) by diffusible substances has recently been the subject of increasing interest (1-2). Such hormonelike factors may also control immune responses *in vivo*, but a rigorous test of their role depends on the availability of highly purified agents: this would permit both the identification of the producing and responding cells, and a detailed characterization of the endocrine circuits that might regulate the immune system. Based on the use of one or another *in vitro* assay, the partial purification from cell-free conditioned media of some factors of rodent or human origin has been reported: thus, a macrophage-derived lymphocyte-activating factor (3), a growth factor for T lymphoblasts (TCGF or interleukin 2)¹ (4), a T cell replacing or "helper" factor (TRF) (5, 6), a costimulator needed for T cell-mediated cytotoxic responses (7), and diffusible mediators of T cell suppressor functions (8, 9), have been characterized to varying degrees.

TCGF, defined as a factor required for the continued proliferation of activated T cells, is present in the conditioned medium of concanavalin A (Con A)-stimulated mouse spleen cell cultures. Recent studies have suggested that TCGF may be important for amplifying lymphocyte populations in response to antigenic challenge; for instance, it has been shown that partially purified TCGF is sufficient for the persistent clonal growth of various T lymphocyte classes (10, 11). While purifying a lymphocyte product that induces macrophage plasminogen activator production (12), we observed that certain protein fractions from conditioned media were enriched in the ability to sustain T lymphoblast proliferation. We describe here the purification of the responsible molecule and the initial characterization of some of its biochemical and biological properties.

Materials and Methods

Male BALB/c and athymic (nude) BALB/c mice were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass. Sephadex G-10, Sephadex isoelectric focusing, DEAE-Sephacel, and SP-Sephadex were obtained from Pharmacia Fine Chemicals, Piscataway, N. J.; hydroxylapatite was purchased from Bio-Rad Laboratories, Richmond, Calif.; aquacide type II was obtained from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.; ampholines were purchased from LKB Instruments, Inc., Rockville, Md.; [³H]thymidine was purchased from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y. (sp ac 17.5

* Supported in part by grant CA-08290 from the U. S. Public Health Service, grant BC316 from The American Cancer Society, and Public Health Service Pathobiology Training Grant GM-07245.

¹ *Abbreviations used in this paper:* Con A, concanavalin A; FCS, fetal calf serum; IEF, isoelectric focusing; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SRBC, sheep erythrocytes; TCGF, T cell growth factor; TRF, T cell replacing factor.

Ci/mM); Dulbecco's minimal essential medium and fetal bovine serum were purchased from Grand Island Biological Co., Grand Island, N. Y.; acrylamide, bisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, and ammonium persulfate were obtained from Eastman Kodak Co., Rochester N. Y.; Con A, crystallized three times, was purchased from Miles Laboratories, Inc., Elkhart, Ind. Tissue culture plasticware was obtained from Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif. All other reagents were of the highest grade commercially available.

Preparation of Conditioned Medium. Male BALB/c mice (15–20 g) were used throughout. In a typical experiment, 500–1,000 spleens were collected, and a suspension of single cells was prepared by pressing the tissue through a fine mesh screen into Dulbecco's medium. The cell concentration was adjusted to 2×10^7 cells/ml in serum-free medium; Con A (5 μ g/ml) was added and the suspension was distributed in 50-ml aliquots into 150-mm plastic tissue culture dishes. Incubation was at 37°C in an atmosphere of 95% air, 5% CO₂. After 2 h of incubation, the cells had settled and adhered to the plate; the supernate was carefully removed and discarded and fresh serum-free medium (without Con A) was added. Medium was collected and replaced at 24-h intervals for a total of 72 h, clarified by centrifugation (500 *g* for 10 min at 4°C), and stored at –20°C, (fraction I). Yield was 64 liters/2,500 spleens.

Hydroxylapatite Chromatography. A slurry of hydroxylapatite was prepared in 20 mM potassium phosphate, pH 7.4, packed into 2.5 × 9.5-cm columns (50 ml volume), and overlaid with a 3-ml layer of Sephadex G-10. The conditioned medium was processed through the first purification steps in batches of 16 liters. After thawing and centrifugation (1,000 *g* for 10 min at 4°C), 2 liters were applied to each column at a flow rate of 60 ml/h. After loading, the column matrix was expressed; the matrices from eight columns were pooled, resuspended, and poured into one large column (8.5 cm²). TCGF eluted in ~600 ml when the column was washed with KPO₄ (20 mM, pH 7.4; fraction II).

DEAE-Sephacel Chromatography. The resin was suspended and washed three times with 0.5 M KPO₄ buffer, pH 7.8, and packed to form a 2.2- × 6-cm column (22.5 ml vol). The column was equilibrated with 20 mM KPO₄, pH 7.8. Fraction II (~600 ml) was adjusted to pH 7.8, and applied at a flow rate of 30 ml/h. The column was washed with 150 ml of 20 mM KPO₄, pH 7.8, and TCGF was eluted using a linear gradient of KPO₄ (0.02–0.35 M, pH 7.8; 450 ml total, flow rate 15 ml/h). The active fractions were pooled and frozen (fraction III).

SP-Sephadex Chromatography. The gel was swollen for 2 h on a boiling water bath (at neutral pH), and was washed several times with 0.5 M NaCl in Na citrate buffer 20 mM, pH 3.5. A 2.5- × 6.5-cm column (35 ml volume) was packed and equilibrated with 20 mM Na citrate, pH 3.5. Fractions II from four preparations were pooled and dialyzed against 20 mM Na citrate, pH 3.5. The precipitate that frequently appeared during dialysis was removed by centrifugation (15,000 *g* for 20 min at 4°C) and the supernate was loaded onto the column at a rate of 30 ml/h. The column was washed with 150 ml of 20 mM Na citrate, pH 3.5, followed by 150 ml of 50 mM NaCl in the same buffer. TCGF activity was then eluted in 100–150 ml of 0.4 M NaCl in citrate buffer, pH 3.5 (fraction IV).

Preparative Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Except where noted, all SDS gels were performed under nonreducing conditions. Fraction IV was dialyzed against 1:10 phosphate-buffered saline (PBS), concentrated 10-fold in aquacide, dialyzed against 1:10 PBS, lyophilized, and resuspended in 1.5 ml of 1% SDS, 20% sucrose, 0.02% bromophenol blue, and applied to a polyacrylamide column consisting of 10 ml of a 15% acrylamide separating gel and 2 ml of a 3% stacking gel, using a Savant gel electrophoresis apparatus and the buffer system of Laemmli (13). The column was cooled with circulating tap water and electrophoresis was at a constant current of 15 mA for 16 h. The proteins emerging at the bottom of the column were continuously removed with electrode buffer and collected in 10-min fractions (1 ml). The active fractions (total volume, 12 ml) were pooled (fraction V). Aliquots of fraction V were mixed with an equal volume of double-strength sample buffer and electrophoresed in an 11% polyacrylamide slab gel, using the buffer system of Neville (14). One lane of the gel was cut into 1-mm slices, eluted in PBS for 18 h at 4°C, and assayed for TCGF activity. When the active material had been localized, the corresponding segments in the rest of the gel were pooled and eluted as above (fraction VI).

Isoelectric Focusing (IEF). This was performed in a horizontal layer of Sephadex. An aliquot

of fraction V was made 10% in Triton X-100, and the sample was then mixed with 1.8 g of Sephadex IEF to give a final slurry of 35 ml. Ampholines (pH 2.5-6) were added to a final concentration of 2% and the slurry was spread in a gel tray. The tray was transferred to a cooling plate (4°C), electrophoresed for 28 h at a constant current of 6 mA, and then sectioned into 21 slices. The gel content of each slice was transferred to a small column (1 × 4 cm), and the pH was determined after adding 1 ml of H₂O. The columns were then eluted with PBS, and each sample was dialyzed against PBS and assayed for biological activity.

Assay for TCGF Activity. Mouse spleen cells were seeded at 1×10^6 cells/ml in 60-mm tissue culture dishes in Dulbecco's medium supplemented with 10% heat-inactivated (56°C for 30 min) fetal calf serum (FCS), 1×10^{-5} M 2-mercaptoethanol, and Con A (3 µg/ml) as described (15). After 4 d, the nonadherent cells were resuspended by gentle pipetting, collected, and washed twice with medium by centrifugation. The cell suspension was adjusted to a density of 4×10^5 cells/ml in Dulbecco's 5% FCS and 50-µl aliquots were dispensed to individual microculture wells (Microtest II plates). Appropriate dilutions of samples to be tested were prepared in Dulbecco's 5% FCS, and 50 µl were added to the cultures. After 24 h of incubation, microplate cultures were pulsed with 1.7 µCi of [³H]TdR. The cultures were harvested 4 h later onto glass fiber strips using a Brandell cell harvester and [³H]TdR incorporation was determined by liquid scintillation counting in Liquifluor-toluene. TCGF activity was expressed in units by testing serial (twofold) dilutions of active fractions: 1 U was defined as the minimum amount of TCGF required to double the [³H]TdR incorporation observed in untreated control cultures. Alternatively, a "TCGF-dependent" cell line (alpha RL δ was a generous gift of Dr. M. Palladino, Memorial Sloan-Kettering Cancer Center) was used: 1×10^4 cells in 50 µl minimal essential medium and 5% FCS were plated in microculture wells, and dilutions of samples to be tested were added in 50 µl medium. After 24 h, 1.7 µCi [³H]TdR was added, and the cells were collected 4 h later as described above.

Assay for TRF. Antibody synthesis was measured as described by Mishell and Dutton (16). Spleen cells from BALB/c *nu/nu* mice were resuspended in minimal essential medium supplemented with 5% FCS to a density of 5×10^6 cells/ml and distributed into 35-mm plates; 50 µl of 0.5% sheep erythrocytes (SRBC) was added as antigen to each culture. After 2 d of incubation with gentle rocking, the fractions to be tested were added. The cultures were incubated for an additional 2 d and the cells were harvested and assayed for the antibody production in the Jerne plaque assay as modified by Mishell and Dutton (16). The results are expressed as the number of lytic plaques formed by 10^6 cells.

Protein Assay. The protein content of samples was measured using a Bio-Rad assay (17). Alternatively, when the protein concentration was below the level of sensitivity of this assay (e.g., in fraction VI), samples were subjected to SDS-PAGE, and the protein bands were visualized by a modification of the silver staining technique (18), quantitated by densitometry, and estimated by comparison with known amounts of protein standards (ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor).

Nude Mouse Spleen Cells Stimulation. BALB/c *nu/nu* mouse spleen cells (1×10^6 cells/ml) were seeded in 100-µl aliquots in microtest plates in Dulbecco's medium supplemented with 5% heat-inactivated FCS; Con A (0.5 µg/ml), fraction VI TCGF (2 ng/ml), or TCGF plus Con A were added to some of the cultures. After 4 d, the cells were pulsed for 4 h with 1.7 µCi [³H]TdR and harvested onto glass fiber strips (see above).

Results

Purification of TCGF

HYDROXYLAPATITE CHROMATOGRAPHY. TCGF was quantitatively removed from conditioned medium by passage through hydroxylapatite gel, and was eluted upon washing the column with a low salt buffer (Table I, fraction II).

ANION-EXCHANGE CHROMATOGRAPHY. The material eluted from hydroxylapatite was applied to a column of DEAE-Sephacel. This column was developed with a KPO₄ gradient (Fig. 1). TCGF activity eluted from the resin between 120 and 200

TABLE I
Purification of Mouse TCGF

Fraction	Volume	Total protein	Total units	Specific activity	Yield
	<i>ml</i>	<i>mg</i>		<i>U/mg</i>	%
I	64,000	1,920	44,000	22.9	100
II	2,400	551	30,769	55.8	70
III	500	108	20,000	185	45.4
IV	125	6.4	13,195	2,061	29.9
V	12	0.84	10,000	11,904	27.7
VI	16	0.08	5,500	68,750	12.5

For determination of protein recovered in each fraction, bovine serum albumin was used as a standard in the Bio-Rad assay. Proteins in fraction VI were determined by densitometry after SDS-PAGE and staining with silver; dilution of known amounts of protein standards ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor was used for comparison.

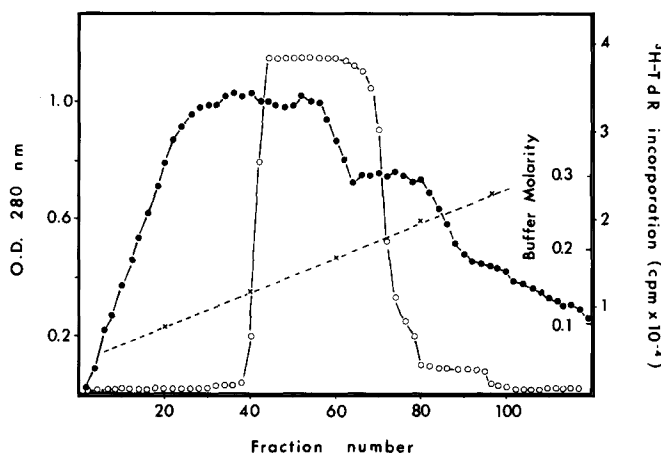


FIG. 1. Purification of TCGF. Chromatography on DEAE-Sephacel: 3.5-ml fractions were collected; the protein content (●) was determined and 5- μ l aliquots were assayed for TCGF activity (○).

mM KPO_4 ; the active fractions were pooled (Table I, fraction III) and stored at $-20^\circ C$.

CATION-EXCHANGE CHROMATOGRAPHY. This was performed on columns of SP-Sephadex as described in Materials and Methods. The column was washed with the adsorbing buffer and then developed by stepwise increases of NaCl (Fig. 2). TCGF activity eluted at 0.4 M NaCl, whereas the bulk of the proteins applied remained bound to the column. The active fractions were pooled (fraction IV), and lyophilized.

PREPARATIVE SDS ELECTROPHORESIS ON CYLINDRICAL POLYACRYLAMIDE GEL. Fraction IV (1.5 ml, 6 mg protein) was applied to a 15% polyacrylamide gel, and the electrophoretic effluent was assayed directly for activity (appropriate controls indicated that cell viability and response were unaffected at final SDS concentrations of $<0.002\%$). TCGF activity was recovered in fractions migrating according to an apparent M_r of 20,000–30,000 (Fig. 3; Table I, fraction V).

PREPARATIVE SDS ELECTROPHORESIS ON POLYACRYLAMIDE SLAB GEL. Aliquots from fraction V pool were applied to an 11% gel. After electrophoresis, the gel lane was cut in 1-mm segments, and the slices eluted into PBS. TCGF activity was recovered as a

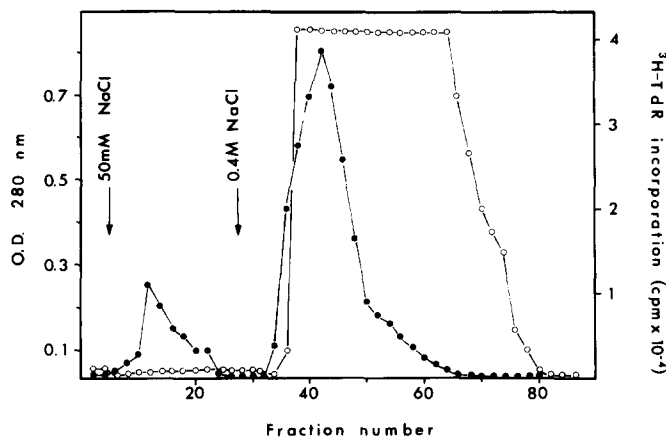


FIG. 2. Purification of TCGF. Chromatography on SP-Sephadex C-25; 3.5-ml fractions were collected; protein content (●) was determined, and 5- μ l aliquots were assayed for TCGF activity (○).

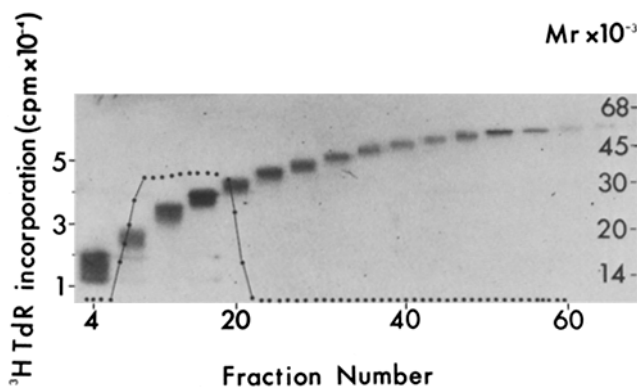


FIG. 3. Preparative SDS-PAGE of fraction IV. Aliquots eluting from the cylindrical gel were assayed for TCGF (●), and electrophoresed in as 12.5% SDS polyacrylamide gel; the illustration shows the Coomassie Blue staining pattern of this analytical slab gel. TCGF activity was recovered in fractions 8-20.

single peak, migrating with an apparent M_r of 23,000 (Fig. 4). The final yield of TCGF purified from 64 liters of conditioned medium was 80 μ g, with an overall recovery of 12.5%; from the increase in specific activity, it can be estimated that a 3,000-fold purification was achieved (Table I).

Biochemical Characterization of Purified TCGF. When applied to an analytical SDS slab gel and evaluated by the highly sensitive silver staining method (18), purified TCGF migrated as a single band with apparent M_r of 23,000 (Fig. 5b), corresponding to the peak of biological activity (Fig. 4). Under reducing conditions, the relative mobility of this band did not change when compared with standard proteins (Fig. 5a), suggesting that TCGF is composed of only one polypeptide chain. Although migration as a single band is expected from the final purification steps employed, the fact that its relative migration did not change upon reduction suggests that this band consisted of few, and perhaps only a single molecular species.

The isoelectric point of TCGF was determined by IEF as described in Materials

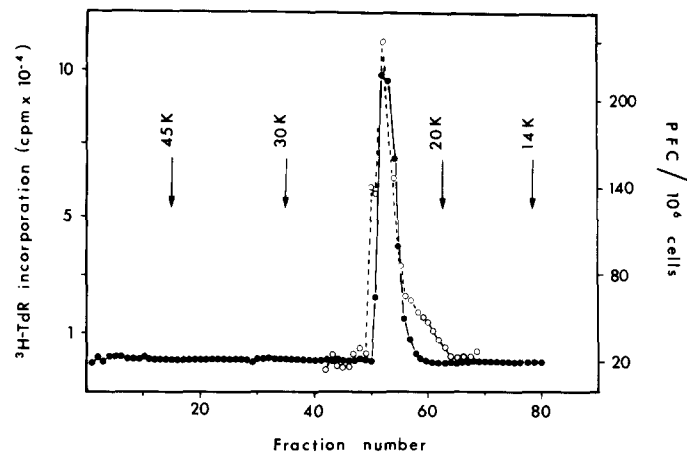


FIG. 4. Preparative SDS-PAGE of fraction V. Slices of the polyacrylamide gel were extracted and assayed for TCGF activity (●) and TRF activity (○).

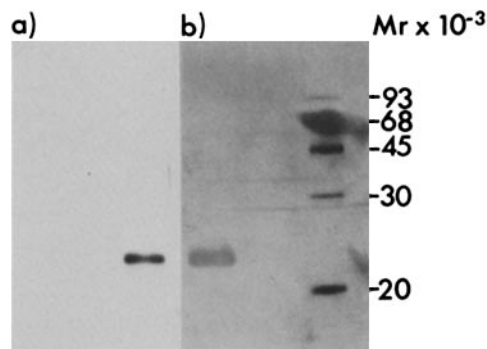


FIG. 5. SDS-PAGE of mouse TCGF. (a) Fraction VI TCGF run under reducing conditions (2.5% 2-mercaptoethanol in sample buffer). (b) Left lane: fraction VI TCGF, nonreduced. Right lane: molecular weight standards: phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α -lactalbumin.

and Methods. TCGF activity was recovered as a single peak in the pH range of 3.9 to 4.4; peak activity migrated with an isoelectric point of 4.0–4.1 (Fig. 6).

Biological Activity of Purified TCGF

DOSE-RESPONSE CURVE. The dose-response curves for the effect of purified TCGF on the proliferation of Con A-induced mouse spleen cell lymphoblasts, and of a TCGF-dependent mouse lymphoid cell line are given in Fig. 7. The data show, first, that these cell populations are equally responsive to stimulation by TCGF, and second, that 1 ng/ml is the lowest concentration at which activity is detectable. Assuming an M_r of 23,000, this indicates that TCGF can stimulate lymphocyte proliferation at a concentration of 4×10^{-11} M.

STIMULATION OF MITOGENESIS IN NUDE MOUSE SPLEEN CELLS. As it has been shown that lymphoid cells from nude mice do not respond to Con A (19), we assessed the ability of our purified TCGF preparation to restore the response of nude spleen cells to lectins. Fig. 8 demonstrates that purified TCGF induced a marked proliferative response; this response was observed in the absence of added lectin, and was enhanced

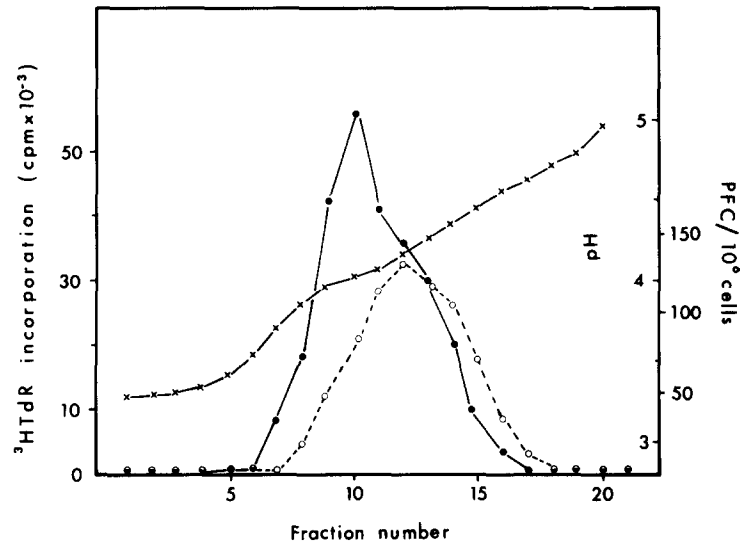


FIG. 6. IEF of partially purified TCGF (fraction V). TCGF activity (●), TRF activity (○).

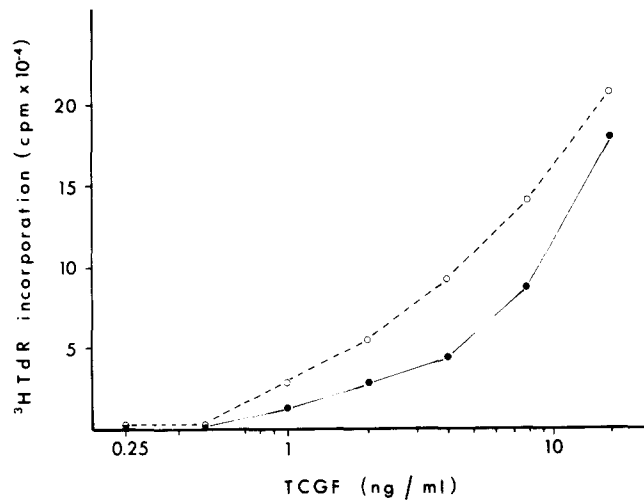


FIG. 7. Dose-response curve for the effect of fraction VI TCGF on lymphoblasts (●) and on a TCGF-dependent cell line (○).

when both Con A and TCGF were present. Comparable results were obtained with spleen cells from normal or nude BALB/c mice, both in the presence and absence of serum.

TRF ACTIVITY. We assayed fractions at different stages of purification for TRF activity to determine whether purified TCGF could substitute for the T lymphocyte requirement in antibody formation by cultured spleen cells from nude mice. Figs. 4 and 6 show that TCGF and TRF activities could not be separated on the basis of molecular weight or of isoelectric point. The minimal amount of fraction VI TCGF that gave detectable stimulation of plaque formation was 2–3 ng/ml (1×10^{-10} M); this was in the same range as the minimal dose required in the TCGF assay. Taken

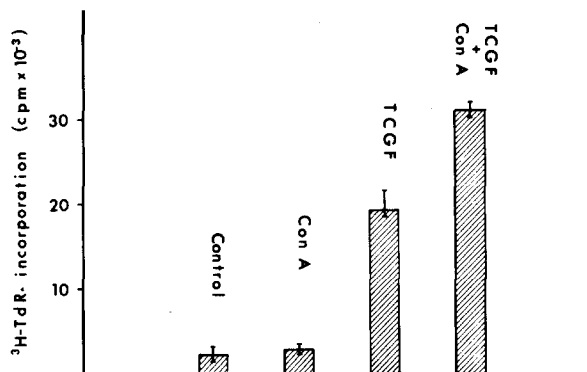


FIG. 8. [³H]TdR incorporation by 1×10^6 BALB/c nude mouse lymphoid cells: a 4-hr [³H]TdR pulse was given after 96 h of incubation under indicated conditions, i.e., tissue culture medium alone, or supplemented either with Con A (0.5 μ g/ml), fraction VI TCGF (2 ng/ml), or fraction VI TCGF (2 ng/ml) plus Con A (0.5 μ g/ml).

together, these results suggest that purified TCGF is either identical to TRF, or that it stimulates the generation of TRF activity in the cultures.

Discussion

We report here the purification of a factor necessary for the sustained proliferation of mouse T lymphoblasts. The activity is associated with a single protein band migrating in SDS-PAGE with an apparent M_r of 23,000, and has been purified 3,000-fold from serum-free conditioned medium. To our knowledge, this is the most extensive purification of TCGF reported to date. Mouse, rat, and human TCGF have been characterized and partially purified by Watson et al. (4), and Gillis et al. (20), although no information was provided as to the specific activities of the materials obtained. Mier and Gallo (21) have reported a 425-fold purification of human TCGF, starting from a conditioned medium in which 90% of the protein consisted of added bovine serum albumin; hence the purification factor obtained by them was only ~40-fold, based on endogenous proteins of the culture. The purification protocol described here introduces three steps that have not been used in other attempts at preparative purification of TCGF: (a) adsorption and elution from hydroxylapatite gave excellent concentration, accompanied by significant purification; (b) cation-exchange chromatography on SP-Sephadex at low pH produced a good increase in specific activity; and (c) taking advantage of the stability of TCGF in presence of SDS, two consecutive electrophoretic runs in polyacrylamide gels were convenient final purification steps. All of these procedures yielded excellent recovery of biologically active material and thus contributed significantly to the successful purification of TCGF.

Although it is clear that the material we have isolated is highly purified, the precise degree of purity has not yet been completely assessed. The fact that electrophoresis under reducing conditions yielded only a single band with minimal change in migration suggests that the bulk of fraction VI TCGF consists of a single or very few protein species. Nevertheless, additional procedures will be required to determine more rigorously the extent to which the single stained band may be contaminated with one or more protein species. If we assume that the TCGF preparation obtained is at least 90% pure, and given the apparent M_r of 23,000, it can be seen from the

dose-response curve of Fig. 7 that TCGF activity is detectable at concentrations as low as 4×10^{-11} M, with the maximum effect being achieved at 10^{-9} M. This concentration range is comparable to that found for the effects of polypeptide hormones in most biological systems and it supports the view that some of the soluble factors present in lymphoid cultures can be considered mediators of a typical endocrine system.

Given the variety of biological responses elicited by spleen-conditioned medium, it is of interest to ask which can be attributed to this purified protein. Confirming previous results obtained with less purified material (4), our purest fractions support the multiplication of primary and early passage T lymphoblasts and of established T cell lines. TCGF was also directly mitogenic to primary cultures of both nude and normal mouse spleen cells, in the presence or absence of serum. Our results therefore contrast with those of Gillis et al. (19), who found a requirement for the presence of Con A in order to observe a mitogenic effect of partially purified TCGF; our finding agrees with the report of Bodeker et al. (22), who noted a direct stimulatory effect of TCGF on different populations of T lymphocytes, and discussed possible reasons for such discrepancies. Finally, the highly purified protein was equipotent in the TCGF and TRF assays; but, although this might be taken to suggest that TCGF interacts directly with B lymphocytes to stimulate antibody production, the TRF assay is performed with unfractionated nude mouse spleen cells, and the possibility that the response to TCGF is mediated indirectly by effects on other, unidentified subpopulations, has not been excluded. This emphasizes the need for both purification of reagents and responding cell populations to unravel the endocrine networks of the immune system.

Summary

Mouse T cell growth factor was purified from the serum-free conditioned medium of lectin-stimulated spleen cells. A 3,000-fold purification was achieved with a final yield of 12%. The purified protein, with an apparent M_r of 23,000 by sodium dodecyl sulfate polyacrylamide gel electrophoresis, was active at concentrations of 4×10^{-11} M, both in the T cell growth factor and T cell replacing factor assays. In addition, purified T cell growth factor alone was mitogenic for spleen cells from both nude and normal mice.

We thank Ms. Patricia Maurides, Atsuko Horiuchi, Karen Kolowski, and Claire Ming-Ko Li for excellent technical assistance.

Received for publication 4 May 1981.

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