

Purification and partial sequence analysis of human T-cell growth factor

(interleukin 2/monoclonal antibody/lymphokines)

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ABSTRACT A murine monoclonal antibody directed against human T-cell growth factor (TCGF) from the JURKAT cell line was used for affinity column purification of the factor. Bound TCGF was eluted nearly quantitatively at low pH, and the recovered factor appeared homogeneous by two-dimensional gel electrophoresis. The molecule is markedly hydrophobic, with a high content of leucine. A single NH₂-terminal sequence of 36 residues was obtained by automated Edman degradation, further supporting the homogeneity of the material. Thus, significant quantities of purified TCGF have been prepared in a single step, making possible detailed analysis of its molecular structure and biological role.

T-cell growth factor (TCGF, also designated interleukin-2) is a necessary signal for the proliferation of activated T lymphocytes (1, 2). The factor also has been implicated in potentiation of immunoglobulin secretion (3-4), T-cell differentiation (5), augmentation of natural killer cell activity (6), and induction of interferon (7). Because it is unclear whether some or all of these activities are attributable to a single molecule, the need for a homogeneous factor with which to define the role of TCGF is obvious. Moreover, TCGF or dysfunctions of its production may be involved in certain pathological states (8, 9), making the availability of large amounts of purified factor useful for clinical studies.

Various approaches have been used for the partial purification of TCGF by biochemical methods (10, 11). In fact, the purification to apparent homogeneity of biosynthetically labeled human TCGF by fractionation with gel filtration and isoelectric focusing has been reported (12). The same process could be used effectively for unlabeled TCGF (13), but it was time-consuming to scale up. A more recent report describing a series of dye matrixes to purify TCGF (14) showed promise but was limited by the number of steps used and a relatively low yield. Ideally, a more selective affinity column, such as one using a specific antibody, would solve the problems associated with scale-up.

Recently, several murine monoclonal antibodies have been prepared against the human TCGF molecule (see Acknowledgment). This report describes the use of one such antibody for the large-scale, one-step purification of JURKAT cell line-derived TCGF. The purified factor, which appeared homogeneous by electrophoretic analysis, was used to determine the molecule's amino acid composition and NH₂-terminal sequence.

MATERIALS AND METHODS

Production of TCGF. TCGF was prepared from a high-producer subclone (J6.8.9.15) of the human leukemia T-cell line,

JURKAT (15). The cells (4×10^6 cells per ml) were stimulated in serum-free Dulbecco's modified Eagle's medium with 1.5 μ g of purified phytohemagglutinin (PHA; HA-16, Wellcome Reagents) and 50 ng of phorbol 12-myristate 13-acetate (PMA; Consolidated Midland, Brewster, NY) per ml. The cell supernatant was harvested after 15-20 hr of incubation at 37°C. It was stored at 4°C (with addition of 0.02% sodium azide) or concentrated 100-200 times by using an H1P5 hollow-fiber cartridge (Amicon).

Biosynthetically radiolabeled TCGF was prepared as described (12) by stimulation of J6.8.9.15 cells in serum-free Dulbecco's modified Eagle's medium containing 40 μ M unlabeled leucine, 65 μ M unlabeled lysine, and 0.5 mCi of [³H]leucine (50 Ci/mmol, ICN; 1 Ci = 3.7×10^{10} Bq) and 0.5 mCi of [³H]lysine (40 Ci/mmol) per ml.

TCGF Assay. TCGF biological activity was determined by the TCGF concentration-dependent stimulation of proliferation (measured by [³H]thymidine incorporation) of a cloned murine cytotoxic T-lymphocyte line (CTLL-2, subclone 15H) (16). The dilution of a sample yielding 50% of the maximum CTLL [³H]thymidine incorporation was determined and divided by that of a standard (1 unit/ml, 50% [³H]thymidine incorporation at about 1:10) to give the concentration of TCGF in units per ml. PHA and PMA at the concentrations that remained after induction had negligible direct or synergistic (with TCGF) effects in the assay.

Monoclonal Antibody to TCGF. Antibodies to human TCGF were raised by three immunizations of a female BALB/c mouse with 4,000 units of TCGF derived from the JURKAT cell line and partially purified by gel filtration (see Acknowledgment). The spleen cells were fused with the NS-1 myeloma cell line, and wells with hybrid growth were screened for reactivity with TCGF by using a solid-phase radioimmunoassay. JURKAT TCGF, purified to apparent homogeneity by gel filtration and isoelectric focusing (13), was added to microtiter wells (Immulon Removawell Strips; Dynatech, Alexandria, VA) at a concentration of 10 biological units (about 30 ng) per 100 μ l of phosphate-buffered saline. After incubation at 4°C for 16-18 hr, the wells were coated with bovine serum albumin (10 mg/ml in phosphate-buffered saline), followed by sequential reactions with undiluted hybridoma cell supernatants and ¹²⁵I-labeled sheep anti-mouse immunoglobulins (8 μ Ci/ng; New England Nuclear). Each hybridoma supernatant also was screened against wells coated with bovine serum albumin alone. Five of the 500 hybridoma-positive wells were specifically reactive to TCGF, and these cells were subcloned by limiting dilution.

Of the subclones that secreted antibody to TCGF, one designated 1H11-1A5 released a particularly high-affinity antibody

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Abbreviations: TCGF, T-cell growth factor; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate.

(as determined by dilution of purified antibody in the radioimmunoassay; unpublished data). This antibody, which was subclass IgG2a, was chosen for preparing an immunoaffinity column. The antibody was routinely purified (>98% pure by electrophoretic analysis) by affinity chromatography of 1H11-1A5 cell supernatants on protein A-Sepharose.

Affinity Chromatography of TCGF. The purified 1H11-1A5 antibody was immobilized (8 mg of 1H11-1A5 antibody per ml of Sepharose) by using CNBr-activated Sepharose 4B. The crude supernatant from stimulated JURKAT cells (or a $\times 100$ – $\times 200$ concentrate of the supernatant) was passed through the column at a flow rate of 100–200 ml/hr, after which the column was extensively washed with 1 M NaCl and 0.5% Nonidet P-40. Tris, which interferes with the determination of histidine on the amino acid analyzer, was removed by washing the column with deionized water. The bound TCGF was eluted with 1.5% acetic acid (pH 2.5). The column has been used repeatedly with no apparent loss of capacity.

To determine if there was residual PMA in the eluted factor, 4 μ Ci of [20 - 3 H(N)]PMA (New England Nuclear) was added to a culture (150 ml) of J6.8.9.15 cells during stimulation. Only 4% of the radiolabel remained in the cell supernatant when the culture was harvested after 16 hr. Of this remaining radiolabel, none was detectable in the pH 2.5 eluate after fractionation on the 1H11-Sepharose column. Thus, $<10^{-5}$ % of the PMA used for stimulation of the JURKAT cells remained in the purified fraction of TCGF. In addition, no detectable PHA remained in the pH 2.5 eluate as assessed by two-dimensional gel electrophoresis.

Protein Determination. The level of protein was determined by the assay of Lowry *et al.* (17) with bovine gamma globulin and bovine serum albumin as standards. The values for purified TCGF were confirmed by quantitative amino acid analysis.

Two-Dimensional Gel Electrophoresis. Purified TCGF (0.9 μ g) was focused on 1.5 \times 120 mm tube gels (6.25%, pH 3–10; Pharmalytes, Pharmacia; 7% acrylamide) followed by second-dimension electrophoresis on 12% acrylamide/NaDodSO₄ gels (18). Protein was visualized by using a silver-staining technique (Bio-Rad). Purified factor was also analyzed on one-dimensional NaDodSO₄/polyacrylamide gel electrophoresis in the presence and absence of reducing agents by the methods of Laemmli (12% and 15% acrylamide) (19).

Amino Acid Composition and NH₂-Terminal Sequence. The amino acid composition of purified TCGF was derived from 24-hr hydrolyses (6 M HCl at 110°C) of 0.5-nmol quantities of the protein, followed by analysis on a Beckman 119CL analyzer. Tryptophan was determined by hydrolysis in 4 M methanesulfonic acid. The NH₂-terminal sequence was determined by using a Beckman spinning-cup 890C sequenator, followed by HPLC analysis of the phenylthiohydantoin-amino acid derivatives. The sequenator used a modified version of Beckman protein program 11-19-78 (0.25 M Quadrol), which included sequential precipitation of the sample with ethyl acetate (twice) and benzene.

Reduction and Alkylation. Purified TCGF (3 nmol per sample) was denatured by dissolving in 8 M guanidine-HCl/0.35 M Tris, pH 8.1/0.0035 M EDTA. After gassing with nitrogen, the samples were incubated with and without 60 nmol of 1,4-dithiothreitol for 4 hr at 37°C. The samples were cooled to 25°C, and 600 nmol of iodo[3 H]acetic acid (202.8 mCi/mmol, New England Nuclear) was added. After 1 hr at 25°C (in the absence of light), the reaction was quenched with 5 μ l of 2-mercaptoethanol. Excess iodoacetic acid was removed by dialysis against 2.5% acetic acid. In the absence of a reducing agent, 0.37 ± 0.18 nmol of iodo[3 H]acetic acid was incorporated per nmol of TCGF. After reduction with dithiothreitol, 2.41 ± 0.31 nmol of iodo[3 H]acetic acid per nmol of TCGF was incorporated.

Radiolabeled TCGF Cellular Binding. The cellular binding of [*leucine, lysine*- 3 H]TCGF purified by affinity chromatography on 1H11-1A5 antibody-coupled Sepharose was determined as described (12).

RESULTS

Immunoaffinity Column Purification. For a large preparative run, an affinity column (2 ml of Sepharose; 8 mg of antibody per ml) coupled with 1H11-1A5 antibody (1H11-Sepharose) bound about 66% of the TCGF biological activity present in crude supernatants of PHA/PMA-stimulated JURKAT subclone 6.8.9.15 cells (Table 1). In contrast, >97% of the activity was recovered in the unretarded fraction of a similar column coupled with nonspecific murine immunoglobulin. Elution at pH 2.5 of the 1H11-Sepharose column resulted in recovery of >95% of the bound activity, giving an overall yield for purified TCGF of 63%. About 2 mg or 130 nmol of factor were recovered from 8 liters of crude supernatant, representing a 150- to 160-fold purification.

The 30–35% of the TCGF activity that was collected in the unretarded fraction of the 1H11-Sepharose column had no apparent affinity for the 1H11-1A5 antibody because <2% was bound to a fresh 1H11-1A5 antibody-coupled column. In contrast, 99% of the TCGF in the pH 2.5 eluate bound upon re-passage over 1H11-Sepharose (after adjustment of the pH to 7.5). Thus, the 1H11-1A5 antibody recognized a determinant available on only two-thirds of the TCGF molecules in the crude supernatant.

Homogeneity of Purified TCGF. When analyzed by two-dimensional gel electrophoresis, with silver staining for detection, the contents of the pH 2.5 eluate migrated as a single spot (Fig. 1A). Treatment with neuraminidase failed to affect the mobility of the spot, thus indicating that the 1H11-1A5 antibody selected for unsialylated JURKAT TCGF (20). Upon one-dimensional NaDodSO₄/polyacrylamide gel electrophoresis, the pH 2.5 eluate migrated as a single band at M_r 14,800 in the absence of reducing agents and at M_r 15,500 in the presence of reducing agents (Fig. 1B). Moreover, when the gel corresponding to a third sample slot (without reducing agents) was sliced and the protein was eluted, all the biological activity (90% recovery) migrated in a position indistinguishable from that of

Table 1. Purification of JURKAT TCGF on the 1H11-1A5 immunoaffinity column

	Volume, ml	Protein,* mg	TCGF activity, units	Recovery, %	Specific activity, units/mg	Fold purification
Cell supernatant	8,180	515	1,032,000	(100)	2,004	(1)
Unretarded fraction and washes	8,300	510	356,000	34.5	698	0.35
pH 2.5 eluate	6.0	2.1	650,000	63.0	310,000	155

*The quantity of protein was determined by the assay of Lowry *et al.* (17).

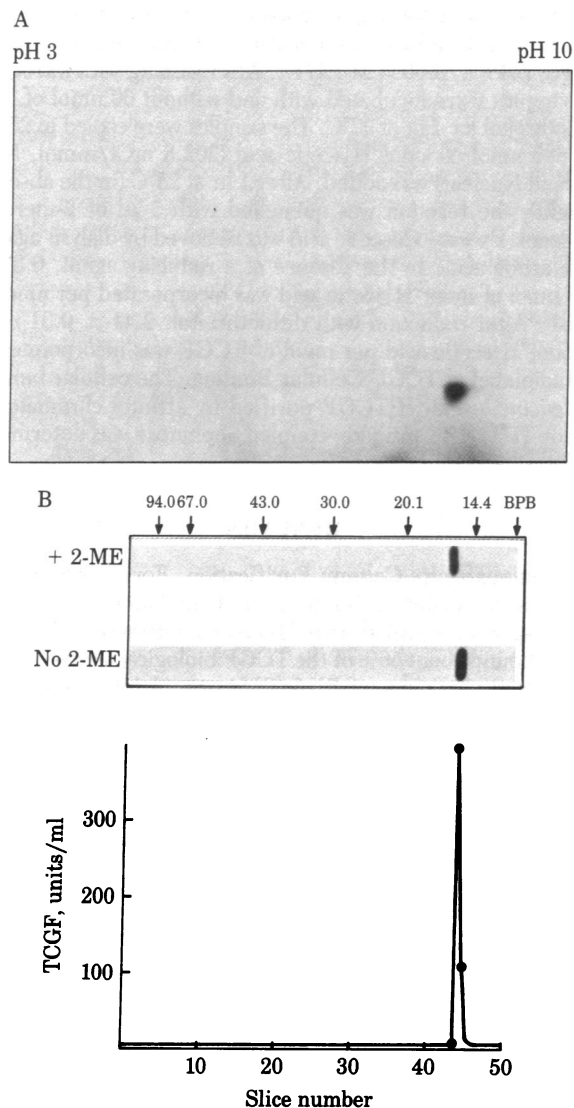


FIG. 1. (A) Two-dimensional gel electrophoresis (pH 3–10) of the pH 2.5 eluate of the 1H11-Sepharose column. Protein was visualized by silver stain. The diffuse stain at the bottom of the gel is due to residual Ampholines. (B) NaDodSO₄/polyacrylamide gel electrophoresis (12% acrylamide, with and without 2-mercaptoethanol (2-ME)) of the pH 2.5 eluate. A third sample slot (no 2-ME) from the same slab gel was cut into 2-mm slices. Each slice was eluted in 1 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum for 48 hr, and the biological activity of the supernatant was determined in the standard CTLL assay. The standards used in calibrating the gel were phosphorylase b (M_r 94,000), bovine serum albumin (M_r 66,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000), soybean trypsin inhibitor (M_r 20,100), and α -lactalbumin (M_r 14,400). BPB represents bromophenol blue, the marker dye.

the silver-stained band (without 2-mercaptoethanol).

As an additional criterion of purity, biosynthetically labeled [*leucine,lysine*-³H]TCGF was purified on the 1H11-Sepharose column and tested for binding to cells known to contain a receptor for the factor (12). Under conditions where the cellular receptors exceeded the number of TCGF molecules, >95% of the radiolabel was bound (Table 2). In contrast, <1% of the label bound to TCGF-nonresponsive B- or T-cell lines. Binding of [*leucine,lysine*-³H]TCGF to the CTLL-2 subclone 15H cells could be prevented by inclusion of a 20-fold excess of unlabeled TCGF purified by either the 1H11-Sepharose column or by gel filtration and isoelectric focusing (13).

Table 2. Binding of [*leucine,lysine*-³H]TCGF by various cell populations*

Cell type	Bound, dpm	Free, dpm	% bound
TCGF-responsive cells			
Murine CTLL-2 subclone 15H	471	23	95.2
Human PHA-stimulated blasts [†]	481	16	96.7
TCGF-nonresponsive cells			
Raji	4	491	0.8
JURKAT	3	493	0.6

*The cells (10×10^6 cells per 200 μ l of RPMI 1640 medium with 1% bovine serum albumin) were incubated with radiolabeled TCGF (500 dpm) at 37°C for 10 min.

[†]PHA-stimulated blasts were prepared from human peripheral blood mononuclear cells by 72-hr stimulation with the lectin. Blast cells were enriched to 95% on a discontinuous gradient of Percoll.

Only very gradual (5–15%), if any, loss of TCGF biological activity in the pH 2.5 eluate occurred upon storage at 4°C for periods of 2–3 months. It did not matter if the material was left at pH 2.5 or if the pH was raised to 7.5. Although small amounts (1–10 units or 3–30 ng per ml) of purified TCGF usually underwent significant adsorptive losses of activity in the absence of carrier molecules (bovine serum albumin or polyethylene glycol 4000), the pH 2.5 eluate at $\geq 250 \mu$ g of TCGF per ml was quite stable.

Amino Acid Composition and NH₂-Terminal Sequence Analysis. The amino acid composition of TCGF (the pH 2.5 eluate) is given in terms of mole percent and in terms of the number of residues expected for a M_r 15,000 protein (Table 3). The latter values would change if a significant carbohydrate component had gone undetected. Because the value of M_r 15,000 agrees well with that found for JURKAT TCGF translated *in vitro* with a rabbit reticulocyte lysate (21), any contribution by carbohydrate is most likely small. In addition, the composition of TCGF purified from the unretarded fraction of the 1H11 column by conventional means (13) was similar to that of the factor in the pH 2.5 eluate (unpublished observations) sug-

Table 3. Amino acid composition of 1H11-Sepharose-purified TCGF

Amino acid	Mole %*	Residues per M_r 15,000 molecule
Aspartic acid	9.2 \pm 0.17	12.0
Threonine	9.0 \pm 0.10	11.8
Serine	5.2 \pm 0.15	6.8
Glutamic acid	13.9 \pm 0.15	18.2
Proline	4.5 \pm 0.22	5.9
Glycine	2.1 \pm 0.09	2.8
Alanine	4.2 \pm 0.11	5.5
Cysteine [†]	2.1 \pm 0.38	2.7
Valine	3.0 \pm 0.29	3.9
Methionine	2.1 \pm 0.28	2.8
Isoleucine	6.3 \pm 0.22	8.2
Leucine	17.4 \pm 0.44	22.7
Tyrosine	2.2 \pm 0.13	2.9
Phenylalanine	4.6 \pm 0.12	6.0
Histidine	2.2 \pm 0.27	2.9
Lysine	8.1 \pm 0.24	10.6
Arginine	3.1 \pm 0.02	4.1
Tryptophan	0.7	0.9
Total	100.0	130.8

* Values (mean \pm standard error) were derived from four independent preparations of TCGF.

[†] Cysteine was determined as cysteic acid or carboxymethylcysteine.



FIG. 2. NH₂-terminal sequence of TCGF from the pH 2.5 eluate.

gesting that sequence heterogeneity, if it exists, is likely to be minor.

The NH₂-terminal sequence of the molecule was determined by using automated Edman degradation (Fig. 2). Repetitive analyses on several independent preparations of the factor yielded identical sequences with no indication of heterogeneity at any of the first 36 positions. The phenylthiohydantoin-amino acid derivative found for position 3 did not correspond to any normal phenylthiohydantoin-amino acid, suggesting that it might be a site of post-translational modification.

Maintenance of Cell Growth. TCGF purified on the 1H11-Sepharose column was equally as effective as crude JURKAT cell supernatants in maintaining the growth of the murine CTLL-2, subclone 15H line (Fig. 3).

DISCUSSION

Interest in T-cell growth factor expanded rapidly after its introduction as a means of maintaining T cells in culture (1, 2). Because of the difficulties in purifying the factor secreted by normal human cells, however, most experiments defining the role of TCGF have utilized crude factor preparations containing a number of biological response mediators. In this report, we describe a means of overcoming these purification difficulties by utilizing a high-producing T-cell line in conjunction with an immunoaffinity column for TCGF isolation.

The specific activity of TCGF in the pH 2.5 eluate and the bioactivity in the original JURKAT cell supernatant (Table 1) can be used to estimate that TCGF made up 0.65% of the initial protein. This significant proportion reflects the high level of factor (average 125 units/ml or 3×10^6 molecules per cell) released by the J6.8.9.15 cells and the relatively low level of soluble protein (60 μ g/ml) in the supernatant. By contrast, the protein content of a typical PHA/PMA-stimulated tonsil cell supernatant contained $\leq 0.01\%$ TCGF (unpublished observa-

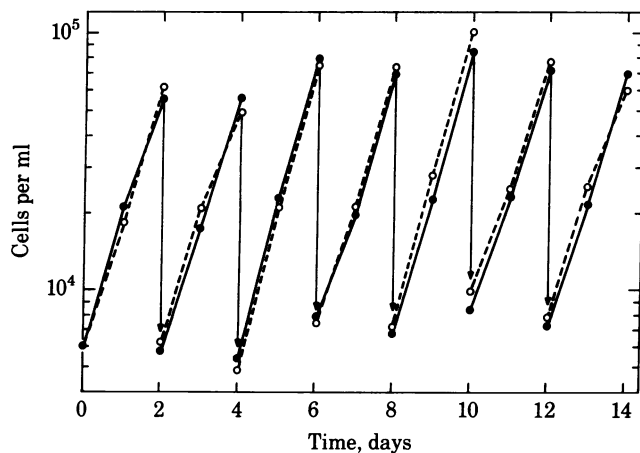


FIG. 3. Maintenance of cell growth. Murine CTLL-2 subclone 15H cells were incubated at the indicated cell density in medium containing TCGF (1 unit/ml) derived from unfractionated cell supernatant of stimulated JURKAT cells (●) or after purification on the 1H11-Sepharose column (○). The cell cultures were split 1:10 into fresh TCGF-containing media every second day.

tion). The crude JURKAT supernatants were thus a highly enriched source of factor.

The structural feature on the TCGF molecule that is recognized by the 1H11-1A5 antibody is absent or masked on about a third of the factor secreted by J6.8.9.15 cells and on the vast majority ($\geq 90\%$) of TCGF secreted by normal human lymphocytes (unpublished observations). These differences in 1H11-1A5 reactivity could be related to the variations in glycosylation that characterize human TCGF (20). In this regard, the binding of immunoaffinity-purified [*leucine, lysine*-³H]TCGF to the antibody was eliminated by treatment with certain glycosidases (unpublished data). Moreover, recent experiments have demonstrated that a purified tryptic peptide consisting of the first eight NH₂-terminal amino acids of TCGF specifically competed with radiolabeled TCGF for binding to the 1H11-column (unpublished data). Because the amino acid in position 3 appeared to have undergone post-translational modification, it seems plausible that glycosylation of this residue is somehow involved in recognition of the factor by the antibody.

Several findings indicate that the protein component in the pH 2.5 eluate was homogeneous and did indeed represent a form of T-cell growth factor. First, a single silver-stained spot was observed on two-dimensional gel electrophoresis (Fig. 1A). The position of the spot was indistinguishable from that of the unsialylated form of TCGF purified by gel filtration and isoelectric focusing (12, 20). In addition, the mobility of the protein component of the pH 2.5 eluate on NaDodSO₄/polyacrylamide gel electrophoresis coincided perfectly with that of the biological activity (Fig. 1B). Second, immunoaffinity-purified, biosynthetically labeled TCGF bound specifically to cells that are TCGF-responsive (Table 2), and such binding could be blocked by competition with unlabeled TCGF prepared from either JURKAT or human tonsil cells and purified by conventional means (13). Third, a single NH₂-terminal sequence was obtained by automated Edman degradation (Fig. 2). Fourth, a low-affinity monoclonal antibody (unpublished data) derived from the same fusion as 1H11-1A5 was equally effective in directly inhibiting (99%) the bioactivity of the pH 2.5 eluate as it was in inhibiting the activity of crude JURKAT-derived or tonsil-derived TCGF (unpublished data). Finally, purified factor from the pH 2.5 eluate was as capable as crude TCGF in maintaining growth of the murine CTLL-2 subclone 15H line (Fig. 3). Moreover, when compared with crude TCGF, the immunoaffinity-purified factor had the same relative activity in the bioassay with either the murine CTLL-2 line or human PHA-stimulated blast cells (unpublished observations). Thus, the dependence of these cells upon a growth factor from stimulated JURKAT cells was satisfied by a single purified protein. At the usual concentration (1 unit/ml) used to maintain the CTLL line, the pH 2.5 eluate described in Table 1 would be sufficient to prepare 650 liters of growth medium.

JURKAT-derived TCGF purified on 1H11-Sepharose had a high content (42%) of hydrophobic amino acids (Table 3) consistent with its observed affinity for hydrophobic chromatography supports (unpublished observations). In particular, there was a high level of leucine (nearly 1 out of 6 residues). Moreover, the number of proline residues observed may place constraints upon the length of α -helical or β -sheet structures.

The amino acid composition data also suggested the presence of two or more cysteines per molecule. Alkylation of purified TCGF with iodo[³H]acetic acid after treatment with and without a reducing agent has indicated that the molecule contains a disulfide bridge. Because the mobility of the molecule on NaDodSO₄/polyacrylamide gel electrophoresis was virtually unaffected by the presence of a reducing agent (Fig. 1B), the disulfide bridge most likely is intramolecular. Moreover, the

disulfide bond is important for maintaining the molecule's biological activity because treatment under strong reducing conditions or reduction/alkylation destroyed most or all of the activity as measured in the TCGF bioassay (unpublished observations).

The NH₂-terminal sequence is characterized by a clustering of hydroxyl amino acids in positions 4–7. In addition, residues 1–10 constitute a relatively hydrophilic region, which is followed by a more hydrophobic, leucine-rich section (positions 12–25). Comparison of this sequence with that of other proteins at the National Biomedical Foundation identified no significant sequence homologies. In a paper that appeared after completion of the work described here, T. Taniguchi and co-workers published the cDNA sequence for human TCGF (22). The amino acid sequence corresponding to their data agrees perfectly with the sequence of the first 36 residues that we derived from the protein (position 3 was identified as a threonine based on the cDNA sequence).

The specific activity of the TCGF in the pH 2.5 eluate (Table 1) was about 2-fold higher than a previous estimate obtained with material purified by gel filtration and isoelectric focusing (12). This discrepancy arose because the original value was largely based on a dye-binding assay (23) with bovine gamma globulin as a standard. However, measurements by the assay of Lowry *et al.* (17) with bovine gamma globulin and bovine serum albumin as standards yielded a 2-fold lower estimate of the quantity of protein for the same sample. This latter value has been confirmed by quantitation on the amino acid analyzer with more stringent conditions than previously used. It was unlikely that the immunoaffinity column yielded a more highly purified factor preparation than did gel filtration and isoelectric focusing because the amino acid compositions derived from TCGF prepared by these two methods agreed very well.

The reagents and procedures described here have been used to prepare centigrams of growth factor. Such large quantities of highly purified TCGF should enable characterization of its complete structure, including identification of those features recognized by the cell-surface receptor. Moreover, such preparations will help in dissecting the role of TCGF in complex immune reactions. As understanding of the contribution of this and other lymphokines increases, the potential for their use in therapeutic manipulation of the immune system should emerge.

Note Added in Proof. We recently have determined that TCGF that binds to the 1H11-Sepharose column has *N*-acetyl-D-galactosamine at-

tached to the threonine in position 3 of the polypeptide chain. TCGF that does not bind to the column may lack the sugar or have additional carbohydrate attached at this position or nearby amino acids.

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