

CHARACTERIZATION OF SOLUBLE FACTORS THAT  
INDUCE THE CYTOLYTIC ACTIVITY AND THE  
EXPRESSION OF T CELL GROWTH FACTOR RECEPTORS  
OF A T CELL HYBRID

BY FRANCOIS ERARD, PATRICIA CORTHESEY, KENDALL A. SMITH,\*  
WALTER FIERIS,<sup>‡</sup> ANDREAS CONZELMANN, AND MARKUS NABHOLZ

*From the Genetics Unit, Swiss Institute for Experimental Cancer Research, CH-1066 Epalinges, Switzerland; the \*Department of Medicine, Dartmouth Medical School, Hanover, New Hampshire 03756; and the <sup>‡</sup>Laboratory of Molecular Biology, State University of Ghent, B-9000 Ghent, Belgium*

There is good evidence that cytolytic T lymphocytes (CTL)<sup>1</sup> are derived from cytolytically inactive precursors (CTL-P). In most experimental systems for the activation of the precursors to CTL, extensive proliferation of the CTL-P is induced. This proliferation depends on T cell growth factor (TCGF or interleukin 2), which acts only on T cells that have become TCGF responsive as a result of their interaction with antigenic stimulator cells. Whether such an interaction is sufficient to induce TCGF responsiveness or whether other signals or factors are required is still controversial (for discussion see 1). In any case, it is not known whether TCGF is required only for growth promotion or whether it has other effects on the maturation of CTL-P. CTL activity can be induced in the presence of a drug that blocks DNA synthesis (2) but it has not been tested whether TCGF is required for CTL activation under these conditions.

Recently, several groups have confirmed the evidence obtained by Raulat and Bevan (3) that the induction of a proliferative response in cell populations containing CTL-P, by exposure to mitogenic lectins and a source of TCGF is not sufficient for the appearance of CTL activity in such populations. The experiments suggest that the maturation of CTL-P requires one or several factors found in the supernatant of spleen cells stimulated with concanavalin A (CS) or of cloned alloreactive T cells stimulated with appropriate stimulators.

To determine the precise requirements for the activation of CTL-P and to elucidate the mode of action of the required signals, it would be useful to establish cell lines with the characteristics of CTL-P. The cell lines derived from CTL described in the literature so far, with the exception of some recently

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<sup>1</sup> *Abbreviations used in this paper:* CIA, cytotoxicity-inducing activity; Con A, concanavalin A; CTL, cytolytic T lymphocyte; CTL-P, CTL precursor; CS, Con A-activated rat spleen cell SN; DMEM, Dulbecco's minimum essential medium; FCS, fetal calf serum; HPLC, high pressure liquid chromatography; IFN, interferon; IL-1, interleukin 1; MAF, macrophage-activating factor; 2° MLC SN, secondary mixed leukocyte culture SN; PEG, polyethylene glycol; pI, isoelectric point; PMA, phorbol-12-myristate-13-acetate; SN, supernatant; TCGF, T cell growth factor.

described clones (4), do not have these characteristics: they do not return to an inactive CTL-P-like state when their growth is arrested by removal of TCGF or the lack of antigenic stimulation (5, 6). But recently we have discovered that hybrids between a mouse CTL line and a rat thymoma, which grow in normal growth medium (i.e., independently of TCGF), can be induced to acquire cytolytic activity when they are cultured in CS. Thus, in some respects these cells resemble CTL-P and we have investigated which factors in CS are required for induction of the hybrids' CTL activity and attempted to determine if TCGF has other functions beside growth promotion. We have found that TCGF together with a second factor controls CTL activity and TCGF receptor expression.

### Materials and Methods

*Hybrid T Cell Line PC60.* The T cell hybridoma PC60. 21.14.4.6 (PC60) was derived from a fusion between a cloned CTL line, B6.1.SF.1, with cytolytic activity against the myeloma S194, and a rat T lymphoma W/Fu(C58NT)D (C58) (7). The cells grow in basic growth medium (mixed leukocyte culture [MLC] medium) (8), with a doubling time of 18 h. Their rate of proliferation is not affected by the addition of CS although such supernatants induce PC60 cells to acquire cytolytic activity (7).

*Supernatants (SN).* (a) SN of Concanavalin A (Con A)-activated rat spleen cells (CS) was prepared by culturing  $3-5 \times 10^6$ /ml spleen cells from OFA rats for 72 h in MLC medium containing 5  $\mu$ g/ml Con A. (b) Serum-free CS was prepared as was CS, but using  $8 \times 10^6$  spleen cells/ml. Its concentrated form was obtained from the material soluble in 40% saturated ammonium sulfate but precipitated by 85% saturated ammonium sulfate. The precipitate was dissolved in a minimum volume of serum-free medium and extensively dialyzed. (c) Secondary mixed leukocyte culture SN ( $2^\circ$  MLC SN) were prepared by culturing, per milliliter,  $10^6$  cells recovered from 14-d-old BALB/c anti-DBA/2 primary MLC with  $4 \times 10^6$  irradiated (2,000 rad) DBA/2 spleen cells (9). The SN was collected after 24 h of incubation. For other experiments,  $10^6$  spleen cells from C57BL/6 mice primed in vivo with DBA/2 cells were restimulated in vitro with  $4 \times 10^6$  irradiated DBA/2 spleen cells/ml (9). The SN of the cultures were harvested at different times (MLC day x SN, SN harvested on day x). (d) SN of phytohemagglutinin-activated human spleen cells were prepared as previously described (10). (e) EL-4 SN containing mouse TCGF was collected after stimulation of EL-4 thymoma cells (kindly provided by Dr. Farrar, NIH, Bethesda, MD) at  $10^6$ /ml for 48 h with 10 ng/ml phorbol-12-myristate-13-acetate (PMA) (11). (f) Jurkat SN containing human TCGF was derived from a high producer clone of the Jurkat T leukemia. The cells were stimulated with 15  $\mu$ g/ml phytohemagglutinin and 50 ng/ml PMA for 18 h at  $37^\circ\text{C}$  in Dulbecco's minimum essential medium (DMEM) containing 4% fetal calf serum (FCS) (12). (g) Purified TCGF from Jurkat SN was obtained as previously described (13). Briefly, TCGF-containing conditioned medium was passed through an immunoaffinity column of 10 mg DMS-3 anti-TCGF monoclonal antibody coupled to 1 ml of Affigel 10 (Bio-Rad Laboratories, Richmond, CA). The column was washed successively with 20 ml each of (a) 1 M NaCl, 10 mM Tris-HCl, pH 7.5, (b) 0.5% Nonidet P-40, 10 mM Tris-HCl, pH 7.5, (c) 10 mM Tris-HCl, pH 7.5, and (d)  $\text{H}_2\text{O}$ . The bound material was eluted with 5 ml 0.2 N acetic acid. It migrated as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% acrylamide) with an apparent molecular weight of 15,500 when 1  $\mu$ g of material was applied and the gel stained with silver nitrate. On high performance liquid chromatography (HPLC), a single protein peak was identified that contained a single amino-terminal amino acid (alanine). TCGF biosynthetically radiolabeled with [ $^3\text{H}$ ]leucine and [ $^3\text{H}$ ]lysine was prepared and purified as previously described (12, 13). It had a specific activity of  $2.23 \times 10^5$  dpm/pmol. (h) Recombinant TCGF of human origin was produced as described (14). Purified material was provided by Biogen SA, Geneva, Switzerland.

*CS Fractionation.* (a) Phenyl-Sepharose affinity chromatography: 1 or 2 ml of concentrated serum-free CS was loaded at  $4^\circ\text{C}$  onto a phenyl-Sepharose (Pharmacia Fine

Chemicals, Piscataway, NJ) column (1.8 × 4 cm) equilibrated with 0.8 M ammonium sulfate, 0.15 M sodium chloride, and 0.01 M sodium phosphate, pH 7.3. The elution was performed at a rate of 0.5 ml/min with 20 ml of the starting buffer and then with a linear gradient formed with 50 ml starting buffer and 50 ml 50% ethanediol in 0.15 M sodium chloride and 0.01 M sodium phosphate, pH 7.3 (15). 5-ml fractions were collected in the presence of 0.25 ml FCS or 0.1 ml 25% polyethylene glycol (PEG) 6000. The fractions were dialyzed against DMEM, ultrafiltered, and tested for biological activities.

(b) Reverse phase HPLC: 1 or 2 ml of concentrated serum-free CS was loaded onto a Vydac C18 column (HPLC Technology Limited, Macclesfield, Cheshire, Great Britain) preequilibrated with 0.1% phosphoric acid, 0.1 M sodium perchlorate, and 10% acetonitrile, pH 2.0. The elution was performed at 1 ml/min by a linear gradient of acetonitrile (10–40% in 5 min) followed by a second linear gradient (40–60% acetonitrile in 20 min). 1-ml fractions were collected and immediately tested for biological activities at 50- and 100-fold dilutions in MLC medium.

(c) Sephadex G100 chromatography: 1 ml of concentrated serum-free CS was applied to a calibrated Sephadex G100 (Pharmacia Fine Chemicals) column (0.72 × 142 cm) equilibrated in 0.15 M sodium chloride, 0.01 M sodium phosphate, 0.2% acetonitrile, pH 7.3. The column had a flow rate of 3.6 ml/h. One ml fractions were collected and analyzed.

(d) Isoelectrofocusing: 1-mm slab gels of 5% acrylamide (PAGPLATE; LKB Instruments, Gaithersburg, MD), containing 2.4% (wt/vol) ampholine, pH 3.5–9.5, were prerun for 10 min at 20 W. Samples of concentrated serum-free CS were then applied via filter paper strips (capacity, 20 μl) and fractionated during a run of 1.5 h at 20 W, followed by 1 h at 10 W. The gels were cut into bands of 0.5 cm which were incubated in 1 ml serum-free MLC medium, containing 0.1% PEG 6000 in dialysis tubes, for 48 h. The eluates were recovered after centrifugation at 3,000 rpm and were ultrafiltered. The pH gradient was determined from pieces of gel incubated in 0.1 M sodium chloride.

*Biological Assays.* (a) Cytolytic assay: CTL activity was measured by a standard <sup>51</sup>Cr-release assay (8). 10<sup>4</sup> <sup>51</sup>Cr-labeled S194 cells and 10 μg/ml Con A were added to effector cells prepared in 96-well, round-bottomed microtiter plates in a final volume of 250 μl MLC medium. After 6 h incubation at 37°C, the plates were centrifuged and 100 μl SN was removed for counting. One lytic unit is defined as the number of lymphocytes required to achieve 50% lysis of 10<sup>6</sup> <sup>51</sup>Cr-labeled target cells in 6 h (8).

(b) TCGF binding assay: The binding of <sup>3</sup>H-TCGF to cell surface receptors was measured as described previously (12). Briefly, the cells were incubated for 2 h in a large volume of TCGF-free MLC medium to remove any bound TCGF molecules. Aliquots of 10<sup>6</sup> of these cells were incubated in serial dilutions of <sup>3</sup>H-TCGF (sp act, 2.23 × 10<sup>5</sup> dpm/pmol) in a total volume of 200 μl for 20 min at 37°C, in 1.5-ml Eppendorf tubes. 0.8 ml of cold MLC medium was added and the cells were centrifuged 20 s at 9,000 g. The concentration of free <sup>3</sup>H-TCGF in the SN was determined. The cells were resuspended in 100 μl MLC medium and centrifuged 4 min at 9,000 g, through 200 μl of a mixture of 85% silicone oil (DC 550 fluid; Serva Feinbiochemica, Heidelberg, Federal Republic of Germany) and 15% paraffin oil (Fisher Scientific Co., Pittsburgh, PA), and the radioactivity remaining in the pellet was counted. The dissociation constant and the number of receptors per cell were calculated from Scatchard plots after correction for nonsaturable binding.

(c) TCGF assay: TCGF activity was quantified, using a TCGF-dependent CTL line in a [<sup>3</sup>H]thymidine incorporation assay (16). Aliquots of 100 μl of MLC medium containing 4 × 10<sup>3</sup> cells were added to serial dilutions of TCGF-containing solutions, in 96-well, flat-bottomed microtiter plates. After 24 h of culture at 37°C and a final [<sup>3</sup>H]thymidine pulse of 6 h (1 μCi/well), the [<sup>3</sup>H]thymidine incorporated in CTL was counted. TCGF activity (U/ml) is calculated from a standardized preparation of TCGF containing 100 U/ml, which on average produces 50% of the maximal response at a 1:100 dilution.

(d) Assay for cytotoxicity-inducing activity (CIA): Serial dilutions of CIA-containing solutions, in some cases complemented with a constant amount of a TCGF-containing solution (see Results), were added to aliquots of MLC medium containing 5 × 10<sup>3</sup> PC60

in 96-well, flat-bottomed microtiter plates. The final volume was 200  $\mu$ l. After 72 h of culture at 37°C, the cytolytic activity of the induced cells against  $10^4$   $^{51}$ Cr-labeled S194 cells was measured in a 6 h assay, in the presence of 10  $\mu$ g/ml Con A (8). To simplify the presentation of results of the fractionation, we have operationally defined as 1 U CIA per milliliter the concentration of this activity which, in the presence of EL-4 SN (at a concentration giving 20–60 U TCGF per milliliter), induces 50% of the maximal specific lysis obtained after culture in 25% CS.

(e) Interferon- $\gamma$  (IFN- $\gamma$ ) assay: IFN activity was quantitated by inhibition of the cytopathic effect of encephalomyocarditis virus on murine L-F2 fibroblast tumor cells, as described (17). IFN activity (U/ml) is calculated by comparison with a reference IFN- $\alpha$  preparation (mouse C243 cell IFN-T139 with  $6.4 \times 10^5$  IU/ml and the NIH mouse reference IFN-B No. G002-904-511). The type was determined from an analysis of the sensitivity of IFN to pH 2 treatment as described (18).

(f) Macrophage-activating factor (MAF) assay: MAF activity was quantitated by measuring the activation of macrophage-mediated cytotoxicity for tumor target cells in a  $^{51}$ Cr-release assay, as described (19). MAF activity (U/ml) was determined by comparison with a standardized preparation of MAF containing 100 U/ml, which on average produced 50% of maximal response at a 1:100 dilution.

## Results

*Reliability of the Assay for Factors Inducing Cytotoxicity in PC60.* As we previously reported (7), the PC60 hybrids become cytolytic when they are cultured in the presence of SN from mitogen-stimulated spleen cells. Maximal induction occurs in 3 d. During induction, the cells grow exponentially at the same rate as the uninduced line.

These properties were used to establish an assay that measures the capability of SN to induce the cytolytic activity of PC60 cells. This assay was designed to permit simultaneous analysis of a large number of SN at multiple dilutions. It consists of microcultures in which the solutions to be analyzed are added to a constant number of PC60 cells. This number is chosen so that the cells can grow exponentially during the induction. 3 d later, a fixed number of  $^{51}$ Cr-labeled target cells are added.

In the experiments described here, the solutions assayed had no detectable effects on the growth of PC60 cells, and at the time of target addition, there were  $\sim 10$  times more PC60 cells than added targets. When we collected the PC60 cells induced in microcultures and determined their cytolytic activity on a per cell basis, we found that the conditions described here gave results comparable to the induction protocols used previously (7).

*Induction of CTL Activity in PC60 Hybrids Requires Two Factors.* In the original report on the PC60 hybrids (7), we had shown that supernatants containing high titers of TCGF were not capable of inducing the cells to become cytolytic. When we began to search for the factors responsible for the induction of the cytolytic activity, we were aware of the observation of Kanagawa (20) that in cultures of leucoagglutinin-treated spleen cells, the generation of maximal CTL activity required a source of TCGF as well as other factors. We therefore began to test combinations of different types of culture SN for their effect on PC60 cells and found that SN of EL-4 cells cultured in the presence of PMA, although devoid of measurable inducing activity, enhanced the titer of cytotoxicity-inducing activity (CIA) in CS (Fig. 1) and allowed its detection in this type of SN as early as 4 h after Con A stimulation.

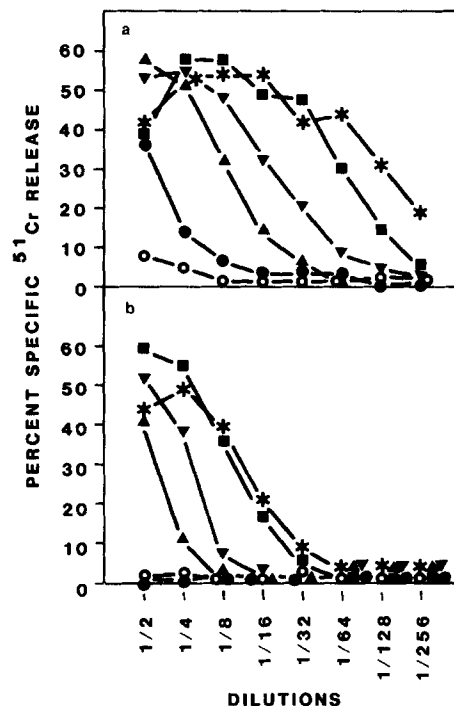


FIGURE 1. Kinetics of appearance of cytotoxicity-inducing activity in CS, and effect of TCGF-containing EL-4 SN on the CIA titer. CS harvested at different times (2 h [O]; 4 h [●]; 8 h [▲]; 12 h [▼]; 24 h [■]; 48 h [\*]) was assayed for CIA on PC60 hybrids (see Materials and Methods), in the presence (a) or absence (b) of 5% EL-4 SN (60 U/ml TCGF).

To rule out that the PMA present in EL-4 SN or the Con A present in CS played any role in the induction of CTL activity in PC60, we tested the effect of these agents at known concentrations: When PC60 cells were induced with CS at concentrations giving a suboptimal induction, the addition of PMA at concentrations  $>0.01$  ng/ml, i.e., 0.1% of the concentration added to EL-4 cells, resulted in a reduction of the induced CTL activity. Lower concentrations had no effect. PMA by itself did not induce lytic activity at any concentration. Con A, at concentrations  $>5$   $\mu$ g/ml (the concentration used in the rat spleen cell cultures producing CS) inhibited PC60 growth. Below this concentration, Con A alone or in combination with various concentrations of EL-4 SN had no effects. A further argument against the role of Con A or PMA in the induction of PC60 is provided by the finding that SN from MLC cultures are able to induce cytolytic activity (Fig. 2). EL-4 SN also enhanced the CIA activity detected in cultures of antigen- or mitogen-stimulated human or mouse spleen cells (Fig. 2).

*CIA and TCGF Can Be Separated by Biochemical Fractionation: CIA Is Not IFN- $\gamma$ .* Fig. 1 shows the kinetics of the appearance of CIA in rat CS, measured on PC60 in the presence (Fig. 1 a) or absence (Fig. 1 b) of EL-4 SN. The recovery of CIA is maximal after 2 d (data not shown). When the SN from MLCs in which responder cells from immune mice restimulated and cultured for 1–5 d were assayed for CIA, in the presence of EL-4 SN, and for TCGF, their titers also reached a maximum after 2 d. CIA activity remained constant until at least day

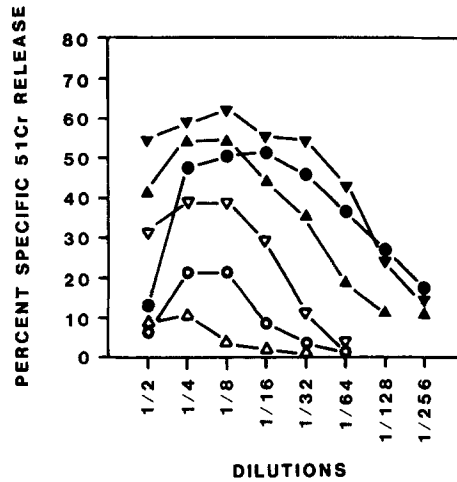


FIGURE 2. Titration of CIA activity in cultures of spleen cells from different species. CIA was assayed in the presence (closed symbols) or absence (open symbols) of 5% EL-4 SN: the SN were obtained from PHA-activated human spleen cells (●, ○) mouse MLC day 5 SN (▲, △), and rat CS (▼, ▽).

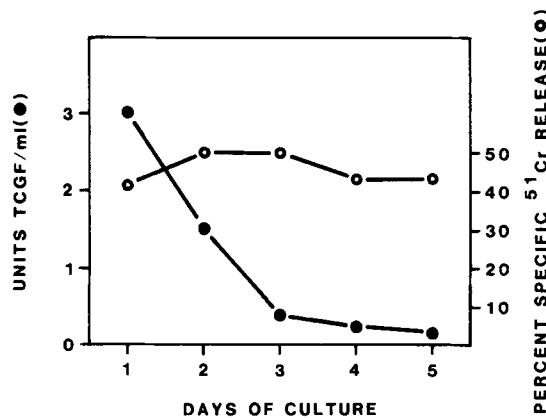


FIGURE 3. Relative amount of TCGF and CIA recovered in MLC SN, 1-5 d after restimulation in vitro of cells primed in vivo. Spleen cells from C57BL/6 mice primed with DBA/2 cells were restimulated with irradiated DBA/2 spleen cells and the cell SN were collected every day and tested for TCGF activity (●) on a TCGF-dependent CTL line, and for CIA (○) in the presence of 10% EL-4 SN (50 U/ml TCGF) on PC60 hybrids.

5 whereas the TCGF titer declined rapidly (Fig. 3). These findings suggested that CIA and TCGF are different molecules.

To conclusively demonstrate this, we fractionated CS by various procedures and analyzed the fractions for CIA and TCGF activity as well as IFN and MAF. For these experiments CS was produced in serum-free medium. This resulted in a twofold reduction of the yields of TCGF and CIA. However, this reduction could be compensated by a twofold increase of the number of treated spleen cells. The serum-free CS was then concentrated 50-100-fold by ammonium sulfate precipitation.

When this material was fractionated on HPLC by a gradient of acetonitrile, at pH 2.0, MAF and IFN coeluted as a single peak, two fractions after the highest concentrations of acetonitrile were reached, while TCGF eluted even later (Fig. 4a). None of the eluted fractions tested by themselves induced more than marginal cytolitic activity in PC60 but, when they were combined with 5% EL-4 SN, CIA became detectable (Fig. 4b). It eluted as a peak at 52% acetonitrile with a long shoulder through the rest of the gradient.

Furthermore, when the HPLC fraction containing maximal CIA activity (fraction 30) was combined with other fractions of the same gradient, induction of cytolitic activity was observed only when CIA was complemented with fractions containing a significant amount of TCGF (Fig. 5).

*Fractionation on Phenyl-Sepharose.* While the yield of TCGF from HPLC was ~70%, <5% of CIA could be recovered. We therefore attempted to separate CIA from other biological activities by another method that also fractionates molecules on the basis of hydrophobicity. The concentrated serum-free CS was applied to a phenyl-Sepharose column equilibrated with buffer containing 0.8 M ammonium sulfate and eluted with a gradient of ethanediol. We obtained the following result (Fig. 6): TCGF eluted at 35% ethanediol and IFN and MAF coeluted at 42% ethanediol. Again, none of the fractions induced significant

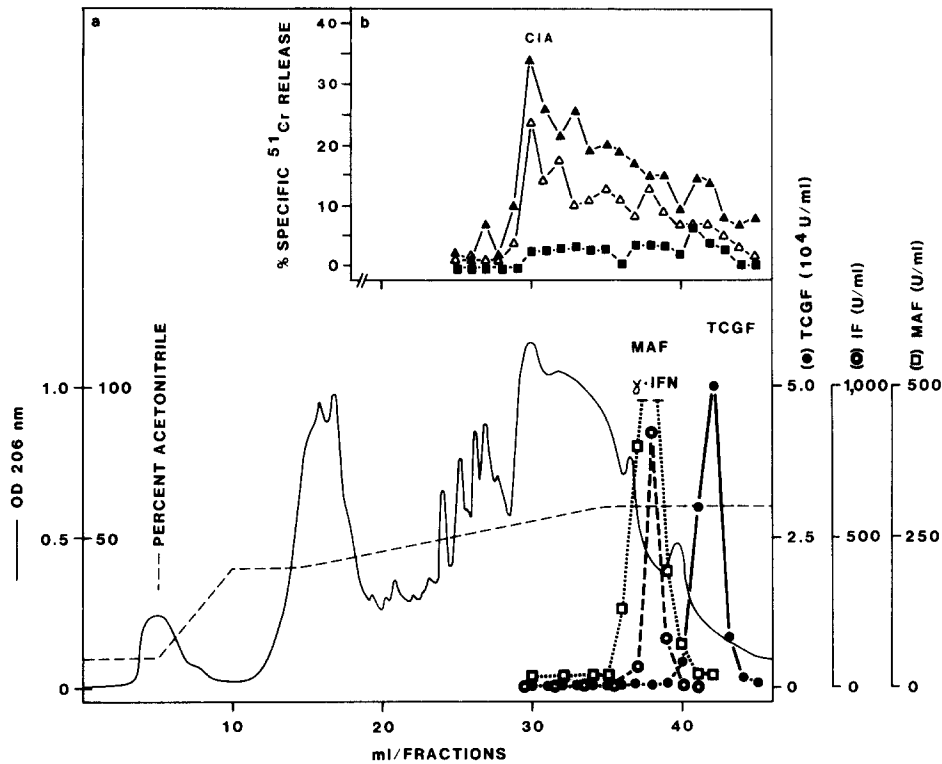


FIGURE 4. Fractionation of concentrated, serum-free CS by HPLC on a C18 reverse phase column. The fractions were assayed for MAF, IFN- $\gamma$ , and TCGF (a) or CIA (b). The latter was tested at 1:50 ( $\blacktriangle$ ) and 1:100 ( $\triangle$ ) dilutions, in the presence of 5% EL-4 SN (see legend to Fig. 1) or at a 1:50 dilution ( $\blacksquare$ ) in the absence of EL-4 SN.

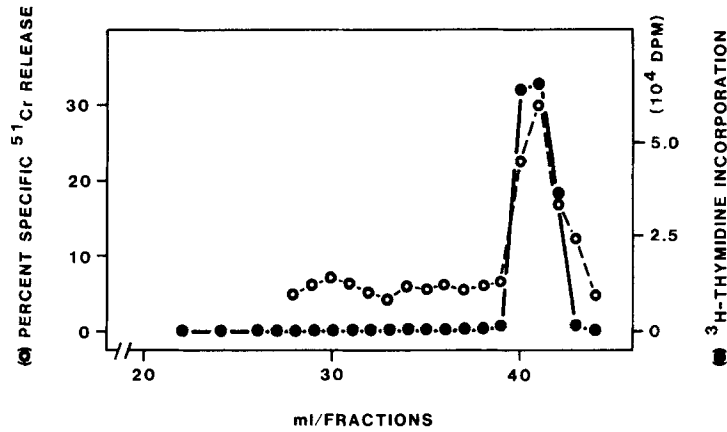


FIGURE 5. Search, in the HPLC fractions, for a second factor, which reconstitutes the CIA found in unfractionated CS. Each HPLC fraction (see Fig. 4) diluted 100-fold, was tested for TCGF (●) as well as for an activity which, in the presence of 1% of fraction 30 (CIA) from the same column, induced PC60 cells to become cytolytic (○).

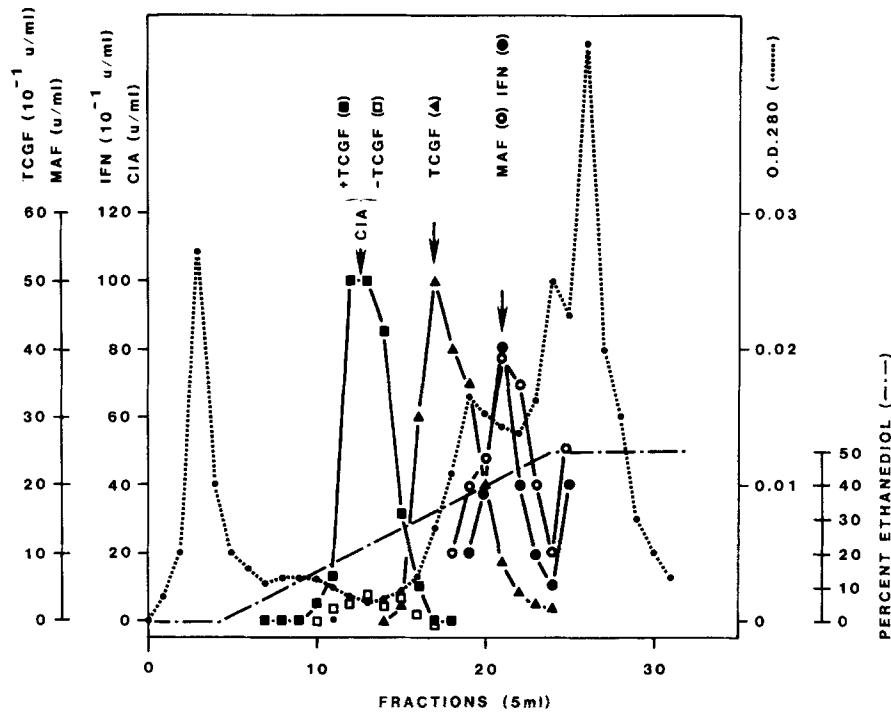


FIGURE 6. Fractionation of concentrated, serum-free CS by phenyl-Sepharose chromatography. CIA was determined in the presence (■) or absence (□) of 10% EL-4 SN.

CTL activity in PC60 when they were tested by themselves. When they were supplemented with 10% EL-4 SN, CIA appeared as a single peak eluting at 20% ethanol. The recovery of CIA was ~40%, that of TCGF ~60%.

*Determination of Isoelectric Point (pI) and Relative Molecular Weight of*



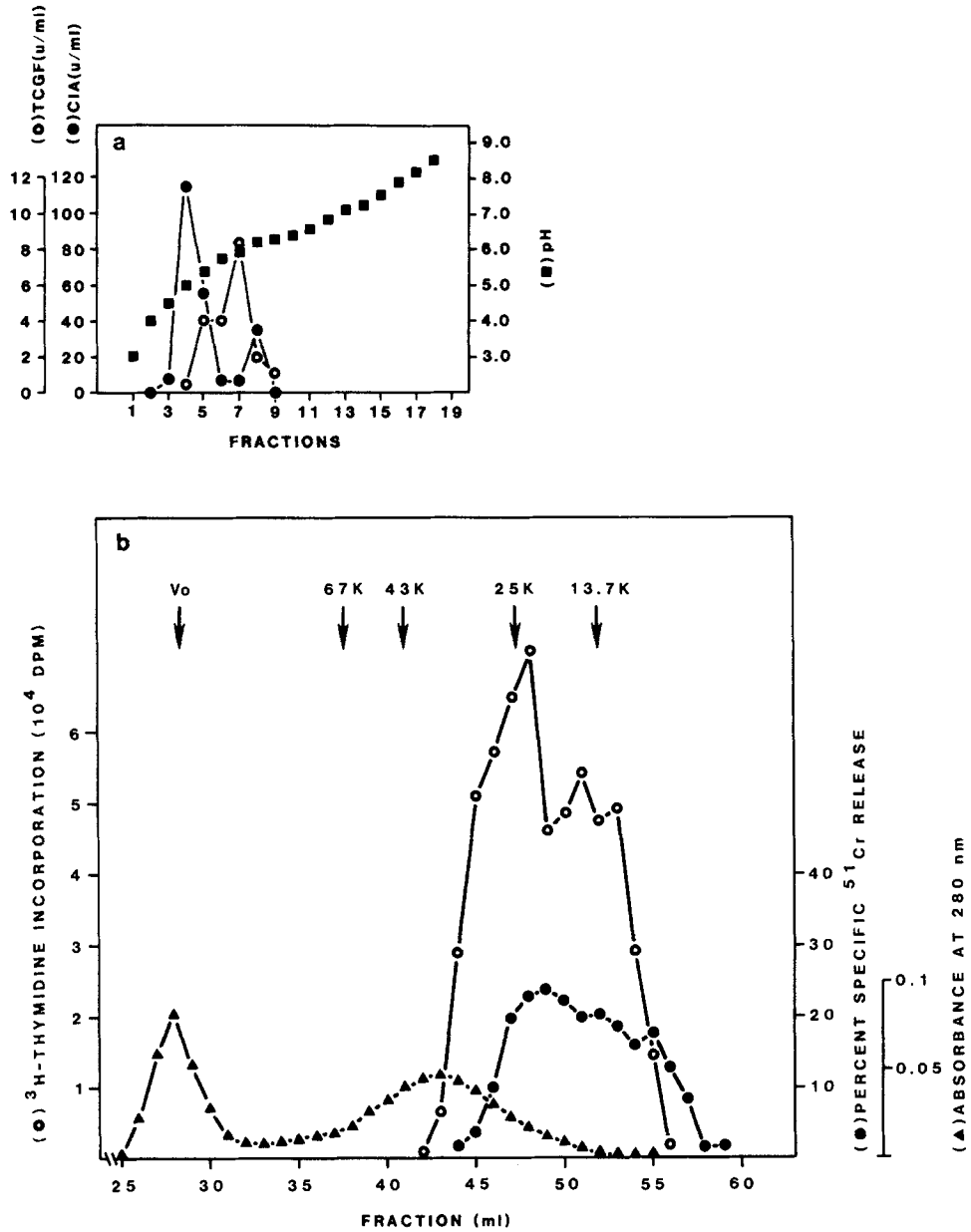


FIGURE 7. Determination of the pI and  $M_r$  of CIA. (a) The relative position of CIA and TCGF found in CS, after focalization on an isoelectrofocusing gel containing ampholines of pH 3.5-9.5. CIA was assayed in the presence of 10% EL-4 SN; b shows the elution profile obtained after chromatography on the same SN on a calibrated Sephadex G100 column. CIA (●) and TCGF (○) activities were determined on dialyzed fractions at a 1:4 dilution.

*CIA*. When serum-free CS was fractionated by isoelectrofocusing on a polyacrylamide gel (Fig. 7*a*), a main fraction of CIA focused around pI 5.0 and a second peak could be observed around pI 6.2. The TCGF activity fell between these values. On Sephadex G100 (Fig. 7*b*), CIA was eluted as a broad peak with an apparent  $M_r$  range of 12,000–18,000, overlapping with TCGF, which eluted with an apparent  $M_r$  of 13,000–23,000.

*Maximal Induction of PC60 Cytolytic Activity Requires TCGF.* Several of the experiments described above suggested that induction of PC60 by CIA depended on the presence of TCGF. To prove that the factor synergizing with CIA was TCGF, we used two sources of purified TCGF. Fig. 8*a* shows that TCGF secreted by the human T cell leukemia line Jurkat and purified to homogeneity by affinity chromatography with monoclonal anti-TCGF antibodies can indeed complement CIA in the induction of PC60 cytotoxicity. The same is true of purified TCGF produced by bacteria carrying a human TCGF-cDNA clone. In the presence of CIA from either phenyl-Sepharose fraction or MLC day 5 SN, TCGF induced the cytolytic activity in a concentration-dependent manner (Fig. 8*b*).

*PC60 Cells Express TCGF Receptors After Culture in CIA and TCGF.* There is evidence that TCGF exerts its biological effects via high affinity cell surface receptors. In view of the finding that TCGF plays a role in the induction of cytolytic activity in PC60 hybrid cells, we quantified the TCGF receptors on PC60 and its parental cell lines, using radiolabeled human TCGF in a direct binding assay. The results of one such analysis are illustrated by Fig. 9. We found that the rat thymoma parental cells did not bind detectable TCGF even after culture in CS. B6.1, the parental CTL line, which is maintained in CS-containing medium, bound ~8,000 molecules of TCGF per cell (Table I). The standard deviation of five independent experiments was 500 molecules per cell.

PC60 cells growing in normal medium did not express any detectable high

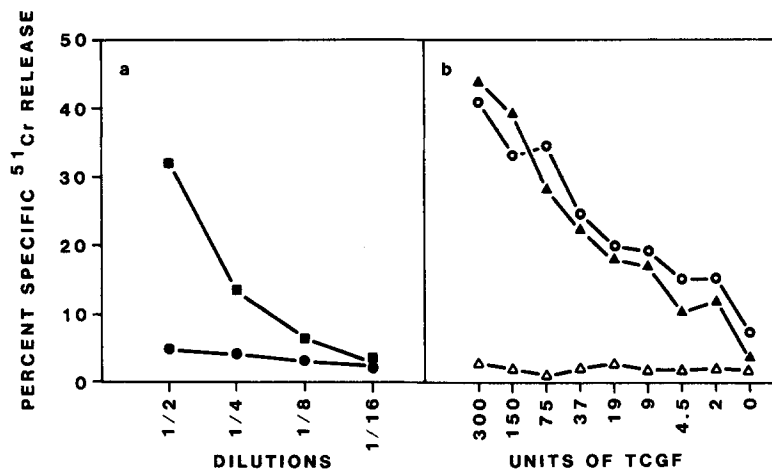


FIGURE 8. Effect of purified human TCGF and recombinant human TCGF on PC60 cytotoxicity induction. (a) Titration of CIA from a phenyl-Sepharose fraction, in the absence (●) and presence (■) of 50 U/ml purified TCGF from Jurkat cells. (b) Titration of the inducing activity of purified recombinant human TCGF alone (Δ) or in the presence of 5 U/ml CIA from either MLC day 5 SN (▲) or phenyl-Sepharose fraction (○).

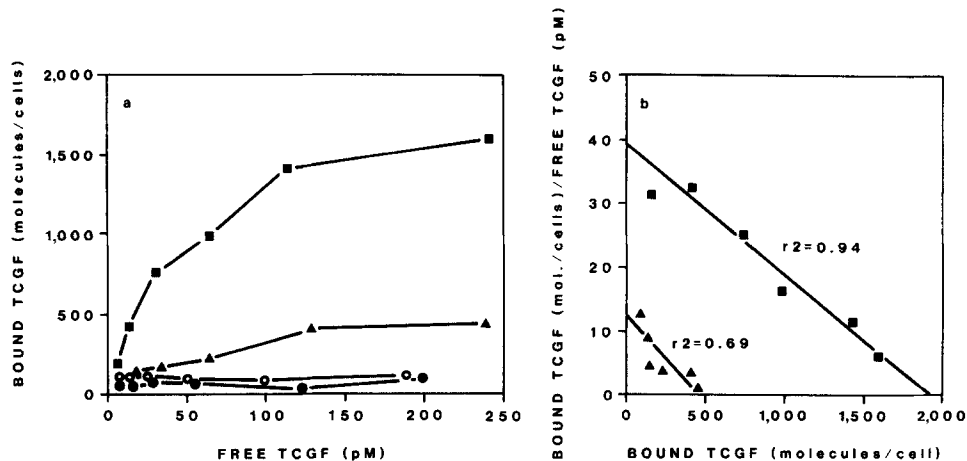


FIGURE 9. Binding of TCGF to induced and noninduced PC60.  $10^6$  PC60 cells cultured for 3 d in either basic medium (●), 45 U/ml affinity-purified TCGF (○), 17% MLC day 5 SN containing 0.5 U/ml TCGF and 3 U/ml CIA (▲) or the combination of the two (■), were incubated for 20 min at 37°C with different doses of labeled  $^3\text{H}$ -TCGF alone or together with a 100-fold excess of unlabeled TCGF (for the determination of nonspecific binding). From the values of TCGF bound we subtracted the machine background and the nonspecific binding, which represented from 0.7 to 0.9% of the radioactivity of the free  $^3\text{H}$ -TCGF (a). The data represented in a were transferred to obtain Scatchard plots (b).  $^3\text{H}$ -TCGF had an estimated specific activity of  $2.23 \times 10^5$  dpm/pmol (15 ng). Thus, 1,600 molecules per cell correspond to 600 dpm of bound  $^3\text{H}$ -TCGF per  $10^6$  PC60 cells.

TABLE I  
Effect of Supernatants on the Expression of TCGF Receptors

Cell type	SN supplements	Cytotoxicity (lytic units/ $10^6$ cells)	TCGF receptors* per cell	$K_d^*$
<i>M</i>				
Parental cells				
B6.1 (CTL line)	CS (25%)	143	8,000	$2.2 \times 10^{-11}$
C58 (T lymphoma)	—	<1	<200	—
	CS	<1	<200	—
Hybrid (PC60)	—	<1	<200	—
	CS	37	1,900	$2.4 \times 10^{-11}$
	MLC SN (25%)	3	1,100	$2.5 \times 10^{-11}$

Cells were cultured, for 3 d in the different SN. Bound TCGF was removed by a 2 h incubation at 37°C in TCGF-free medium and the cells incubated with different doses of purified  $^3\text{H}$ -TCGF with a specific activity of  $2.23 \times 10^5$  dpm/pmol as previously described (12).

\* The dissociation constant ( $K_d$ ) and the number of receptors per cell were estimated from Scatchard plots, after correction for nonsaturable binding.

affinity TCGF receptors for human TCGF, but, after 3 d in CS, the hybrid cells bound 2,000 molecules TCGF per cell. When we cultured PC60 either in the phenyl-Sepharose fractions containing the peak of CIA activity or in purified TCGF, or in a combination of the two, we found that only the cells exposed to both factors expressed detectable numbers of high affinity receptors (Table II).

Con A does not play a role in the induction of TCGF receptors on PC60 cells

TABLE II  
Effect of TCGF- and CIA-containing Supernatants on the Expression of TCGF Receptors by PC60

Experiment*	SN supplements		TCGF	CIA	TCGF receptors <sup>‡</sup> per cell
	EL-4 SN (10%)	MLC (1/1) day 5 SN (17%)			
1	+	-	20	<0.2	<200
	-	+	0.5	3	380
	+	+	20	3	2,000
2	+	-	45	<0.2	<200
	-	+	<0.2	6	<200
	+	+	45	6	1,600

\* Each type of experiment has been done at least twice.

<sup>‡</sup> See Table I.

<sup>§</sup> Purified by immunoaffinity chromatography with anti-TCGF monoclonal antibody.

<sup>†</sup> Fraction obtained from phenyl-Sepharose chromatography of serum-free CS.

since (a) Con A plus TCGF does not induce receptors whereas (b) a combination of EL-4 SN or pure TCGF and MLC day 5 SN or 2° MLC SN does (Tables I and II and Fig. 9).

### Discussion

The PC60 hybrids whose behavior is analyzed here are derived from a cross between a rat thymoma, W/Fu(C58NT)D, which does not express any markers characteristic of mature rat T cells and resembles stem cells or very early thymocytes (W. Jeffrieson, Medical Research Council, Oxford, personal communication), and a mouse CTL line (B6.1.SF.1), which for its growth requires TCGF but no antigenic stimuli (6, 21, 22). The activity of the parental CTL line B6.1 does not seem to be regulated by any factors, since these cells, deprived of TCGF, cease to proliferate but continue to express a constant CTL activity per cell as long as they survive (~40–50 h) (6). When, on the other hand, B6.1 cells are maintained in purified TCGF (i.e., CIA-free) for several weeks, their cytolytic activity also remains constant.

Hybrids between murine CTL and C58 resemble, in many respects, the CTL parent: many of them express Lyt-2 antigens and show specific cytolytic activity that can be inhibited by anti-Lyt-2 antibodies or antibodies against the target antigens (23). The <sup>51</sup>Cr release induced by these cells can be inhibited by EDTA (Conzelmann and Corthésy, unpublished results) and is not mediated by a toxin released into the medium. Some C58 × CTL hybrids depend on TCGF for growth (23).

The particular interest of the hybrid clone investigated here and of similar clones described elsewhere (23) is that their cytolytic activity is controlled by factors in Con A SN (CS). The clone PC60 described here grows independently of TCGF but only expresses cytolytic activity when it is cultured for 2–3 d in CS.

This induction does not require DNA synthesis or cell division (23). We show here that it depends on at least two factors, one of which is TCGF. We have operationally called the other one, cytotoxicity inducing activity (CIA). Purified TCGF by itself has no detectable inducing activity; in contrast, all CIA-containing fractions tested so far can induce some activity at high concentrations but this never reaches the levels induced by a combination of CIA and TCGF.

Fractionation of CIA from CS shows that this activity behaves like a macromolecule with a relative molecular weight of ~12–18 K and pI of 5.0 and 6.2. It is sufficiently hydrophobic to be retained on phenyl-Sepharose or on C18 HPLC columns, but it is less hydrophobic than TCGF or IFN- $\gamma$ . The present data do not allow us to determine whether CIA is due to a single molecular species.

We do not yet have any conclusive evidence concerning the cellular origin of CIA. On the other hand, we found that certain batches of SN from the T lymphoma lines EL-4 or Jurkat contain small amounts of CIA-like material and that CIA can be found in SN from T cell clones cultured with irradiated stimulator spleen cells (20). But, recently, we have detected high levels of CIA-like material in SN from lipopolysaccharide-treated macrophages and in the culture medium of ultraviolet-treated P388D cells, a murine cell line with macrophage characteristics (24). This cell line also produces the lymphokine designated interleukin 1 (IL-1), also known as lymphocyte-activating factor, and CIA resembles IL-1 in many of its biochemical characteristics (25–27).

At present, we have little information about the mode of action of CIA. The fact that TCGF is required for maximal induction of cytolytic activity and that appearance of high affinity TCGF receptors on PC60 hybrids required TCGF as well as MLC SN or CS fractions containing CIA suggests that the induction of CTL activity and of TCGF receptors may be mediated by the same molecules, but we have not yet tested whether the distribution of TCGF receptor-inducing activity in the different fractions is the same as that of CIA. The observation that TCGF itself is required for the induction of detectable high affinity TCGF receptors suggests that uninduced PC60 cells already express either low affinity TCGF receptors or an undetectable number of high affinity receptors. Alternatively, such receptors may be induced by the non-TCGF factor and their expression amplified to a detectable number by TCGF.

We can only speculate on the biological role of CIA and the factor(s) inducing TCGF receptor expression, and on the relevance of the induction of CTL activity in PC60 hybrids for the maturation of normal CTL precursors. But, experiments to compare the properties of PC60 cells with that of homogeneous populations of CTL precursors may now be possible, since Howe and Russel (4) recently described CTL clones whose cytolytic activity depends on the culture conditions and appears to depend on antigen stimulation and the presence of soluble factor(s).

Several other groups have described experimental systems in which the maturation of CTL-P into active CTL depends on factors other than TCGF (3, 20, 28–32) and originally we assumed that those activities were similar or identical to CIA. However, the experiments of Raulet and Bevan (3) and, in particular, those of Kanagawa (20), seem to show that the factor(s) inducing CTL maturation

of lectin-activated spleen cells are not necessary for the acquisition of TCGF responsiveness by the same cells, whereas our results suggest that CIA may act via the induction of TCGF receptor expression, almost certainly a necessary requirement for the capacity to respond to TCGF.

If CIA is required for the expression of TCGF receptors, then we would expect that it plays a role in the proliferative response of T cells to antigenic stimulation. The requirements for such a response are, at present, quite controversial. Hardt and Wagner (personal communication) have obtained results which indicate that there is indeed a requirement for a factor distinct from TCGF in the TCGF response of purified Lyt-2<sup>+</sup> cells to mitogenic lectin, and Hünig et al. (33) have presented evidence in favor of a non-T accessory cell requirement in such responses. Gullborg and Larsson (34), on the other hand, have argued against such a requirement. We expect that the further purification of CIA and the characterization of its mode of action will help to clarify these issues.

### Summary

A rat × mouse T cell hybrid (PC60) proliferates in the absence of T cell growth factor (TCGF) and its cytolytic activity can be induced by culture in mixed leukocyte culture supernatants or concanavalin A-activated rat spleen cell supernatant (CS) to lyse <sup>51</sup>Cr-labeled tumor target cells. To characterize the factor(s) responsible for this reversible induction, serum-free CS was fractionated by reverse phase high performance liquid chromatography and by phenyl-Sepharose chromatography. A cytotoxicity-inducing activity (CIA) was separated from TCGF and macrophage-activating factor/interferon- $\gamma$ . CIA was found to be a macromolecule with an apparent molecular weight of 12,000–18,000 and a pI of 5.0 and 6.2. Its activity on PC60 cells depended on the addition of TCGF. Thus TCGF may have other effects on T cells than the induction of entry into cell cycle.

The number of TCGF surface receptors on PC60 cells was measured using purified <sup>3</sup>H-TCGF. TCGF receptors were undetectable on noninduced cells but appeared during induction. The expression of TCGF receptors was not induced either by TCGF or by CIA-containing supernatants or fractions alone, only by a combination of both. These results show that TCGF plays a role in the regulation of the expression of its own receptors.

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