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Human T-Cell Growth Factor: Parameters for Production

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Using conditioned media (CM) from phytohemagglutinin (PHA)-stimulated peripheral blood lymphocytes (PBL) we observed long-term selective growth of T-cells from normal human donors. This T-cell growth was continuously dependent on addition of a factor called T-cell growth factor (TCGF). The optimal method for preparing highly active CM from single donor PBL involves the addition of mitomycin C-treated B-lymphoblastoid cell lines to the mixture of PBL and PHA. A number of different cell lines greatly augmented the production of TCGF in 18/18 cases. Preparation of plasma membranes from the Daudi cell line could replace the intact cells in the production of TCGF but those from the cell line, Molt-4, could not. Since the cell surface of Daudi possesses HLA-D antigens but not HLA-A, B, and C, and Molt-4 has HLA-A and B and not HLA-D, it is possible that the Ia antigens (HLA-DR_W in man) are important in the release of TCGF. Using this method for growth factor production, an analysis was made concerning the events necessary for lymphocyte activation and the requirements for production and release of TCGF. Removal of PHA 12 hr after incubation had no effect on lymphocyte transformation but decreased TCGF release by 90%. In addition, colchicine and cytosine arabinoside inhibited DNA synthesis but had no effect on TCGF release. Little or no TCGF activity was present after cellular protein synthesis was inhibited by puromycin and cycloheximide. These results suggest that TCGF production: a) requires protein synthesis; b) requires binding of the stimulating agent; c) can occur in a non-dividing cell, probably a terminally differentiated T-cell, without the need for cellular proliferation; and d) needs the assistance of an adherent cell which probably is a monocyte-macrophage. The ability to produce TCGF from single human donors will allow better understanding of the nature and action of TCGF.

Key words: T-cell growth factor, T-cell proliferation, cellular regulation, B-lymphoblastoid cell lines

The discovery of a soluble factor named T-cell growth factor (TCGF) in crude PHA-stimulated leukocyte conditioned media (Ly-CM), made it possible to selectively maintain normal human T-lymphocytes in continuous culture for the first time [1, 2]. This observation prompted attempts to maintain antigen-activated T-cells in continuous culture, and it

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is now evident that antigen-specific cytotoxic and helper T-cells may be grown for extended periods with retention of specificity using TCGF [3–10]. It now appears that the antigen stimulates the initial activation of the T-cell and confers specificity on the cell (ie, the first signal) [2, 11–14]. TCGF, while not involved in the activation process, is required as a second signal to cause the clonal expansion of activated T-cells [8, 12–14].

The method used in our laboratory [1, 2] for the prolonged culture of T-cells resulted in a saturation density of $1.5–2.0 \times 10^6$ cells/ml 4–5 days after an initial concentration of 5×10^5/ml. Subsequent reports by other investigators [8, 11] in both human and animal systems demonstrated that the factor-cell ratio is crucial for growth. In their systems, T-cell growth was optimal between 10^4 and 10^5 cells/ml, and their factor preparations would not sustain growth above 2×10^5 cells/ml. This emphasized the need for more defined methods for TCGF production and for some easy, reproducible way to quantitate factor levels or at least to compare the potency of different factor preparations in a more direct way than increase in cell number. Supernatants from mitogen or antigen-stimulated murine or rat spleen cells were potent sources of TCGF [7, 11]. However, attempts to produce highly active supernatants from mitogen-stimulated human peripheral blood leukocytes from a single donor were largely unsuccessful.

In the present study, procedures were developed that allowed an analysis of the cellular and molecular requirements for the production of human TCGF. In addition, it was found that a rapid assay for ^{3}H-TdR incorporation into cultured T cells, previously described for murine T-cells [11], could be used as a measure of human TCGF activity if used under precisely defined conditions.

MATERIALS AND METHODS

TCGF Production From Multiple Donors

Commercially collected heparinized whole blood was mixed with 0.3 ml of plasmagel (Associated Biomedics Systems, Buffalo, NY) per ml of whole blood and incubated without agitation at 37°C for 1 hour. The leukocyte-rich plasma was collected in such a way as to minimize red blood cell contamination and diluted in an equal volume of serum-free tissue culture medium. This plasma-culture medium mixture was then passed through nylon fiber filled columns (Dupont type-200 nylon, Dupont Chemical, Wilmington, DE) at a rate of 125 ml per min and the eluate from the column was centrifuged for 20 min at 1,000g. The cell pellet was resuspended in a small volume of culture media and mixed with 10–20 cell pellets from other ABO-compatible blood donors. To prepare conditioned media containing TCGF, a leukocyte concentration of 10^6 cells/ml was mixed with 1% human plasma and 0.1 ml PHA-P (Difco, Detroit, MI) per 100 ml of cell suspension and incubated for 72 hours. After 72 hr supernatant fluid was collected, filtered through sterile membranes, and stored at −20°C.

TCGF Production From Single Donors

Leukocyte-rich plasma samples from single donors were kindly provided by the staff of the NIH Blood Bank. Routinely, $5–10 \times 10^8$ leukocytes were obtained after ficoll-hypaque separation [15]. Supernatants to be assayed for TCGF activity were prepared by varying numerous parameters. First, 10^6 peripheral blood leukocytes/ml in RPMI 1640 medium and 5% v/v heat-inactivated fetal bovine serum (Reheis Chemical Co, Phoenix, AZ) were stimulated by PHA-M at 1% v/v (Difco, Detroit, MI) at 37°C. Culture supernatants

were harvested cell-free after 72 hr of incubation and stored at −20°C until assayed for TCGF activity. Within this basic scheme, the following parameters were changed: 1) cell concentrations (2×10^6, 5×10^6, 10^7 cells/ml); 2) media conditions (1% human plasma, 0.5% bovine serum albumin) (Sigma Chemical Co, St. Louis, MO), serum-free; 3) different mitogens at their optimal dose (Concanavalin A, protein A, leuco-agglutinin) (all from Pharmacia Chemicals, Uppsala, Sweden), Pokeweed mitogen, lipopolysaccaride (Difco, Detroit, MI); and 4) times of incubation (12, 24, 48, 72, 96, and 120 hr). Second, adherent cells in PBL were removed by nylon fiber filtration as described above. The resulting lymphocyte populations were greater than 99% pure as judged by esterase strains [16]. Third, 2×10^5 mitomycin C (25 μg/ml) treated cells of a B-lymphoblastoid cell line were added per ml of mixture of PBL and PHA. The cell lines used were Daudi, HSB-2, Molt-4, SB, and Raji (obtained from American Type Culture Collection, Rockville, MD). Finally, PBL were depleted of T-cells by binding to sheep erythrocytes (E-rosette) as previously described [17]. Also, T-cells were lysed using specific antisera and complement [18].

Cultured T-Cells

Long-term cultures of human T-cells were maintained as described previously [1, 2]. These cells were resuspended at 5×10^5 cells/ml in 50% v/v crude Ly-CM prepared from multiple donors. At day 4 or 5, the cells reached their saturation density of $1-2 \times 10^6$ cells/ml and were fed by dilution to 5×10^5 cells/ml. This process was repeated every 4–5 days.

TCGF Assay

TCGF-dependent long-term cultured T-cells were used as the target cells in the assay. The cells to be used as target were in culture for at least 20 days and were at their saturation density for 24 hours. These conditions are necessary to insure that only activated T-cells which need TCGF for growth remain in the cultures. The cells were washed free of growth media and resuspended in media containing 5% FCS. Cells were placed in 96-well microplates (No. 3596 Costar, Inc, Cambridge, MA) followed by serial dilutions of the TCGF sample to be assayed. After 48 hr of incubation, 0.5 μCi of ^{3}H-TdR (specific activity 3 Ci/mM, Schwartz/Mann, Inc, Orangeburg, NJ) were added to each well and cultured for an additional 4 hours. Cultures were harvested onto glass fiber filter strips and ^{3}H-TdR incorporation was determined as previously described [19]. CM were concentrated by Amicon filtration using a UM05 membrane (Amicon Corp, Lexington, MA) or fractionated by DEAE-Sepharose as previously described [14].

Plasma Membrane Isolation

The procedure was modified from one described by Jett et al [20]. Briefly, 5 grams of cells were extensively washed with Earle's balanced salt solution and then 90% glycerol was slowly added in small increments to the cells until a 30–40% final concentration was reached. Lysis buffer (10 mM Tris/HCL, pH 7.4, containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$) equal to 1% of the original volume is rapidly added to the cell pellet and then gently homogenized. The cell-free lysate was centrifuged at 700g for 10 minutes. A crude membrane was obtained by sedimentation on a sucrose cushion at 30,000g for 90 minutes. The crude membrane preparation was then sedimented in a 25–50% sucrose gradient at 100,000g for 16 hours. The gradient fractions were assayed for thymidine 5′-phosphodiesterase [21], a cell membrane marker; β-glucuronidase [22], a lysosomal marker; succinate dehydrogenase [22], a mitochondrial marker; and glucose-6-phosphatase [23], a microsomal marker.

Using this procedure, there was a 15-fold increase in phosphodiesterase activity in the membrane preparation but there was still appreciable lysosomal contamination.

Metabolic Inhibitor Studies

The effect of colchicine, cycloheximide, dexamethasone (Sigma Chemical Co, St. Louis, MO), cytosine arabinoside (Upjohn Co, Kalamazoo, MI), and puromycin (Nutritional Biochemical Co, Cleveland, OH) on the production of TCGF were tested as described in Results. These substances were always used as freshly prepared solutions. Since most of these chemicals were toxic in the TCGF assay, the CM were thoroughly dialyzed against a Tris/HCL, pH 7.4, buffer before assaying for TCGF.

RESULTS

A typical growth factor assay is shown in Figure 1. Cultured T-cells in the presence of increasing concentrations of TCGF incorporated ^{3}H-TdR in a dose-dependent manner. In the crude Ly-CM prepared from multiple donors, inhibition of TCGF-mediated thymidine incorporation was seen at the highest doses of TCGF, but this effect was not observed with TCGF after it had been fractionated on an ion-exchange column [14]. This material then had a sigmoid dose-response curve which is characteristic of several growth factors. The observed inhibition with Ly-CM could be caused by PHA toxicity and, indeed,

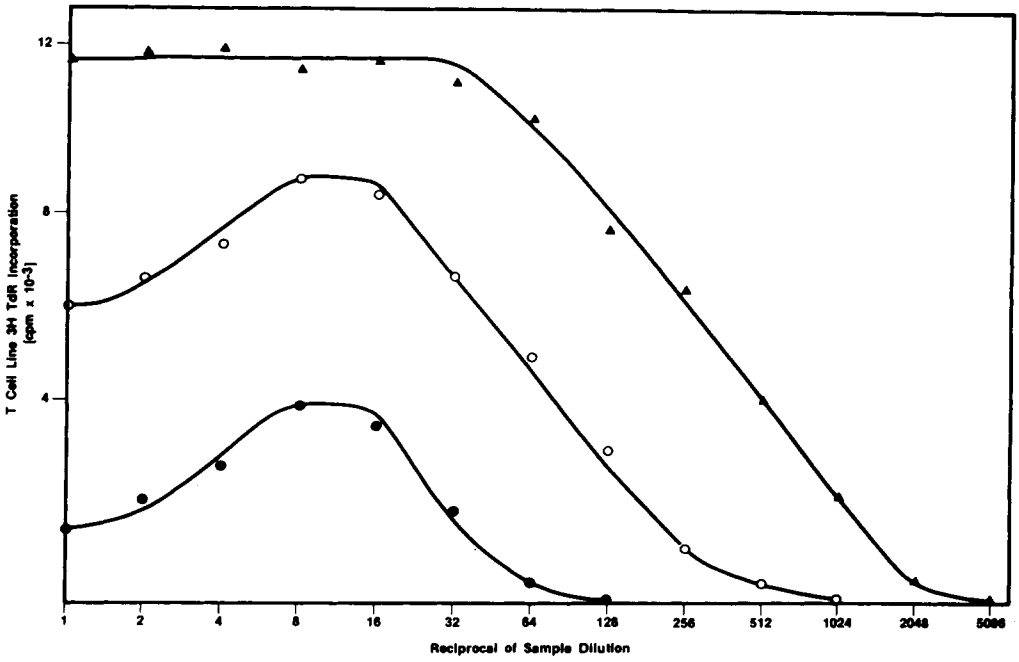

Fig. 1. The TCGF microassay. Human T-lymphocyte cell lines (day 56 in culture; 5 days after last addition of TCGF). ^{3}H-TdR incorporation plotted against the reciprocal of the sample dilution. The samples tested were from 72-hr conditioned media from PHA-stimulated pooled allogeneic peripheral blood leukocytes cultured at 10^{6} cells/ml. Crude CM (solid circles), ammonium sulfate fractionated CM (open circles), and DEAE-Sepharose chromatographed TCGF (triangles) were assayed.

addition of optimal mitogenic concentrations of PHA-P to some TCGF-dependent T-cell lines resulted in a 30–60% inhibition of thymidine incorporation (data not shown). PHA was only weakly mitogenic (2–3-fold more thymidine incorporation into cultured T-cells than controls) in the absence of TCGF and was unable to sustain the growth of T-cells in culture.

Quantitation of TCGF Activity

The ^{3}H-TdR incorporation data generated by stimulating cultured T-cells with serial dilutions of a standard TCGF preparation was plotted on probability paper as a percentage of the maximum ^{3}H-TdR counts incorporated [11, 24]. The sample dilution that corresponded to the 50% level of the maximum incorporation could be defined as 1 unit/ml of TCGF activity. In a similar manner, other TCGF-containing media can be compared to the standard, and a value in units can be determined [11]. For example, the data displayed in Figure 1 was plotted on probability paper (Fig. 2). The factor preparations were calculated as 1 unit/ml, 7.8 units/ml, and 18.5 units/ml for the crude Ly-CM, the ammonium sulfate fractionated sample, and the TCGF after ion-exchange chromatography, respectively. The repeated use of the same TCGF standard allowed comparison of data obtained from different assays. All the remaining data presented is expressed in units/ml as compared to the same TCGF standard.

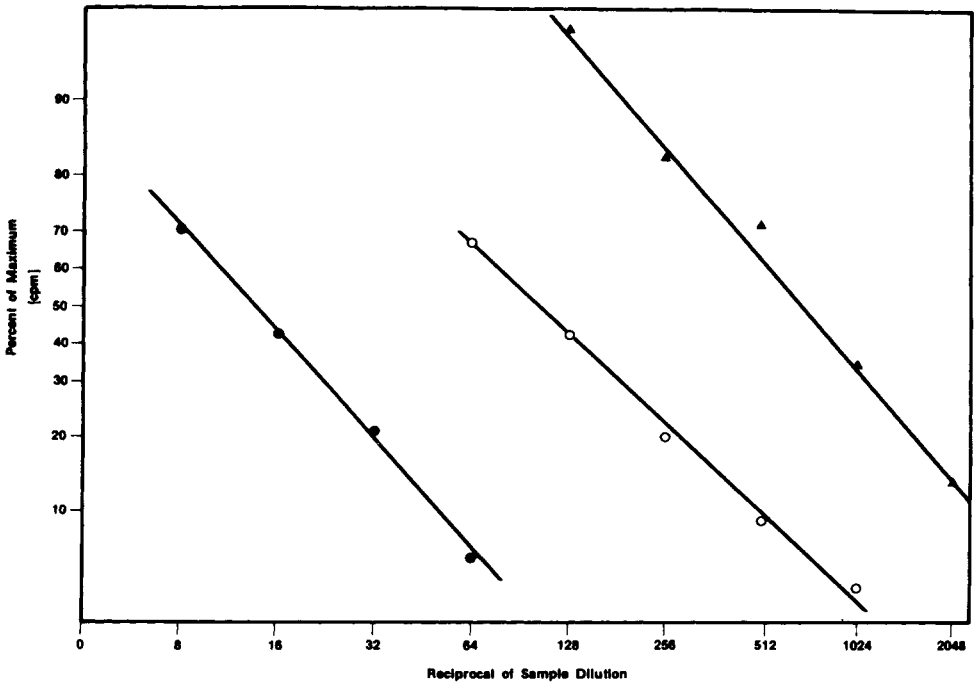

Fig. 2. Probit analysis of the TCGF microassay data. The data presented in Figure 1 were plotted by probit analysis (a value of 100% is given to the cpm of ^{3}H-TdR incorporated at the highest dose TCGF; all other data points are a percentage of the maximum). If the 50% value of the crude CM is assigned a value of 1 unit/ml, then the ammonium sulfate fractionated sample is 7.8 units/ml, and the chromatographed sample is 18.5 units/ml.

TCGF Production From Leukocytes From Single Donors

Attempts to generate CM containing TCGF activity from PBL obtained from individual donors are summarized in Table I. Using 18 different samples, little or no TCGF activity was produced. Varying the time of incubation did not appreciably alter the results. Although supernatants harvested after 24 and 48 hr of incubation stimulated the incorporation of more ^{3}H-TdR than 72 hr, they were not able to maintain the growth of T-cells even after concentration by Amicon filtration and could therefore not be considered positive for TCGF. The removal of adherent cells by nylon fiber filtration, use of other mitogens such as Con A and pokeweed, and the fractionation of inactive CM by ion-exchange chromatography to remove most of the mitogen [14] did not result in the recovery of TCGF activity (Table I). CM generated from leukocytes pooled from multiple donors were always much more active than CM from single donors.

Another approach to produce TCGF from leukocytes from a single donor was suggested by the work of Oliver and his colleagues [25]. They demonstrated that the requirement for the presence of pooled lymphocytes from many donors in assisting human T-cells to become cytolytic for autologous leukemic blast cells [26] could be replaced by using Daudi, a lymphoblastoid cell line. When PBL from single donors were stimulated with PHA in the presence of Daudi at a concentration of 2×10^5 cell/ml, TCGF production was almost as efficient as production using multiple donors (Table II). Addition of mitomycin-treated Daudi cells to a mixture of pooled lymphocytes from 15 donors and PHA led to a substantial increase in TCGF activity. PBL cultured with Daudi cells in the absence of PHA produced little TCGF. Daudi by itself or stimulated with PHA did not produce TCGF. Pokeweed mitogen could replace PHA-M but Con A could not.

TCGF Production Kinetics

To determine the time course of production and release of TCGF, conditioned media were harvested from replicate plates after various times of incubation and then assayed for TCGF (Table III). The kinetics of production were approximately the same for both the multiple and single donor methods. Only minimal activity was present after 12 hr, after which production rapidly increased, reaching a peak at 72 hr. The amount of TCGF in the CM significantly declined after 96 and 120 hr of incubation.

This decline in TCGF activity could be a result of the release of inhibitors of TCGF or nonspecific proteolytic enzymes. To examine this, CM obtained after 72 hr of incubation was diluted twofold with CM harvested after 120 hr incubation or with fresh tissue culture media and then assayed in a microassay for TCGF (Fig. 3). Both samples had equivalent amounts of TCGF, making the presence of an inhibitor in the 120 hr CM unlikely.

Role of Lymphoblastoid Cell Lines in the Stimulation of TCGF Production

In an attempt to better understand the nature of the stimulus supplied by Daudi cells, a number of lymphoblastoid cell lines were tested for their ability to stimulate production of TCGF (Table IV). Molt-4 and HSB-2 were negative and three B-cell lines, SB, Raji and Daudi were positive. Supernatants from these latter cell lines could not replace the need for the cells themselves in the stimulation of TCGF production, suggesting that cell-to-cell contact is necessary. A partial purification of plasma membranes from Daudi and Molt-4 was accomplished by hypotonic lysis of glycerol-loaded cells followed by differential centrifugation. In the case of Daudi cells, but not Molt-4, this crude membrane preparation could replace the need for intact cells in stimulating TCGF production (Table IV). The

TABLE I. T-Cell Growth Factor Production From Human Leukocytes

Cell source of PBL[a]	Samples tested	Mitogen[b] source	Time of incubation (hr)	TCGF Activity[c] (units/ml)
10–20 pooled donors	32	PHA	72	0.8–1.7
10–20 pooled donors	6	none	72	0.0–0.2
Individual donors	18	PHA	24	0.0–0.3
Individual donors	18	PHA	48	0.0–0.3
Individual donors	18	PHA	72	0.0–0.1
Individual donors	5	Con A	48	0.0
Individual donors	5	Pokeweed	48	0.0–0.2
Individual donors (macrophage depleted)	3	PHA	48	0.1–0.3

[a]Peripheral blood leukocytes were isolated as described in Materials and Methods. Macrophages were removed by nylon fiber columns.
[b]PHA-M (1% v/v), Con A (5 μg/ml), and Pokeweed mitogen (15 μg/ml) were used at their optimal mitogenic concentrations.
[c]Cell-free CM were harvested after the indicated time of incubation. The CM were assayed for TCGF activity as described in Materials and Methods.

TABLE II. Generation of Human T-Cell Growth Factor

Responding lymphocyte source[a]	Samples tested	Stimulation source[b]	TCGF Activity[c] (units/ml)
MNC from 15 pooled donors	3	PHA	0.89–1.20
MNC from 15 pooled donors	3	PHA + Daudi	1.30–1.55
MNC	18	PHA	0.00–0.20
MNC	18	PHA + Daudi	0.84–0.96
MNC	3	Pokeweed + Daudi	0.70–0.86
MNC	3	Con A + Daudi	0.00–0.18
MNC pretreated with anti-T-cell sera + C′	3	PHA + Daudi	0.20–0.35

[a]Mononuclear cells (MNC) were isolated as described in Materials and Methods.
[b]PHA-M (1% v/v), Con A (5 μg/ml), and Pokeweed mitogen (15 μl/ml) were used at optimal mitogenic concentrations. Daudi cells were mitomycin C-treated for 30 min and, after extensive washing, were added to the cultures at 2×10^5/ml.
[c]Cell-free CM were harvested after 72 hr of incubation. The CM were assayed for TCGF activity as described in Materials and Methods.

fact that the membrane preparation can be diluted tenfold while retaining 60% of maximal stimulation suggested that this activity is a membrane-bound activity. Daudi has HLA-D antigens but not HLA-A, B, and C on its cell surface [27], while Molt-4 has HLA-A and B but not HLA-D antigens [25]. This effect, whether related to Ia antigens or not, seemed to be directly involved in TCGF production, since mixing supernatants from Daudi and from PHA-stimulated PBL did not enhance TCGF activity.

Cellular Requirements for TCGF Production

The removal of T-cells using anti-T-cell antisera in the presence of complement markedly reduced TCGF production (Table II), as did the depletion of E-rosette positive cells from the PBL, suggesting that a mature T-cell is required (Fig. 4). E-rosette positive

TABLE III. Kinetics of TCGF Production From Human Leukocytes

Culture conditions[a]	Time of incubation[b] (hr)					
	12	24	48	72	96	120
MNC from 15 pooled donors + PHA	0.0–0.1	0.4–0.6	0.9–1.0	1.0–1.2	0.7–0.8	0.5–0.6
MNC + PHA + Daudi	0.0–0.2	0.6–0.7	0.8–0.9	0.9–1.0	0.5–0.6	0.2–0.3

[a]Mononuclear cells (MNC) were isolated as described in Materials and Methods. PHA-M (1% v/v) and Daudi cells, mitomycin C-treated for 30 min, were added to the cultures where indicated.
[b]Cell-free CM were harvested at the times indicated. The CM were assayed for TCGF activity as described in Materials and Methods and the units were calculated by probit analysis using a highly active CM as an arbitrary standard of 1 unit/ml.

cells were able to produce only about 30% of the activity produced by PBL, suggesting that another cell type is also required. To test for the role of adherent cells, nylon fiber columns were used to separate PBL into adherent and non-adherent fractions. Adherent cells (10^5/ml) did not produce detectable TCGF. The addition of 5×10^4 adherent cells to 10^6 E-rosette positive cells resulted in the reconstitution of all the activity found with stimulation of PBL. Human thymocytes, even with the addition of adherent cells, were unable to produce TCGF.

Macromolecular Requirements for TCGF Production

Studies to determine the relationship between DNA synthesis and TCGF production were performed using the mitotic inhibitors colchicine (1 μg/ml) and cytosine arabinoside (10 μg/ml) (Fig. 5). These inhibitors markedly reduced the incorporation of ^{3}H-TdR into DNA but had little effect on the TCGF production. The PHA-stimulated control cultures incorporated over 30,000 cpm of ^{3}H-TdR, while none of cultures containing inhibitors incorporated over 800 cpm; yet these cultures still produced 80% of the TCGF produced in the control. The use of inhibitors of protein synthesis, cycloheximide (50 μg/ml) and puromycin (10 μg/ml), virtually abolished the production of TCGF (Fig. 5). That this inhibition was due to a lack of protein synthesis and not to cellular toxicity was indicated by the finding that the cells could resume making TCGF after the removal of puromycin (data not shown). The removal of PHA after 12 hr of incubation markedly reduced the amount of TCGF production in a 72-hr incubation (Fig. 5).

DISCUSSION

The discovery that CM obtained from PHA-stimulated peripheral blood leukocytes could facilitate the long-term growth of T-lymphocytes [1, 2] has permitted the development of cloned T-cell lines which retained specificity after many months in culture [3–10]. It became apparent that the growth kinetics of cultured T-cells differed in several laboratories [8–11], suggesting that the TCGF-T-cell ratio was important in maintaining growth. It then became important to determine more precisely the cellular and molecular requirements for TCGF production.

First, it was necessary to develop an assay system that permitted a simple and rapid quantitation of TCGF levels in many preparations. A microassay based upon ^{3}H-TdR incorporation in factor-dependent cultured T-cells stimulated by CM containing TCGF, first

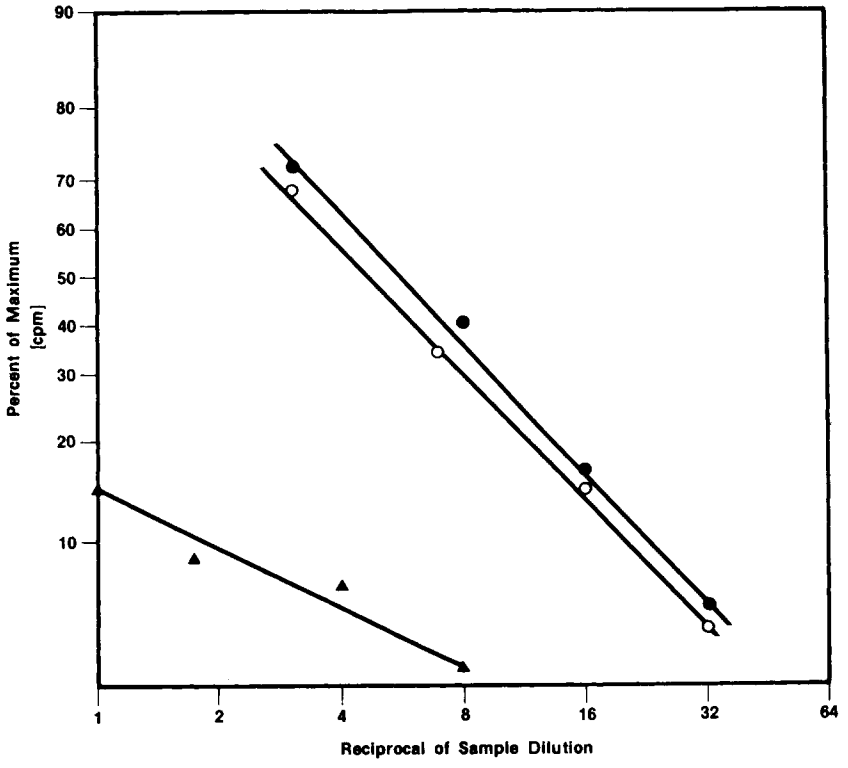

Fig. 3. Lack of inhibitors of TCGF in Ly-CM. Conditioned media for TCGF assay were prepared as described in Materials and Methods. Activity in units was determined by assaying against the standard in Figure 1. TCGF 1:1 dilution with RPMI (solid circles), TCGF 1:1 dilution with 120-hr Ly-CM (open circles), and 120-hr Ly-CM (triangles) by itself were assayed.

TABLE IV. Lymphoblastoid Cell Enhancement of Human TCGF Production

Addition to PBL[a]	HLA Antigens[b]	TCGF Activity[c] (units/ml)	
		+PHA	−PHA
None	–	0.1–0.2	0.0
SB	A,B,D	0.7–0.9	0.0
HSB-2	A,B	0.1–0.3	0.0
Molt-4	A,B	0.0–0.3	0.0
Molt-4 plasma membrane	A,B	0.0	0.0
Daudi	D	0.8–1.3	0.2–0.3
Daudi plasma membrane	D	0.6–1.0	0.1
Daudi plasma membrane (1:10 dilution)	D	0.5–0.8	0.0

[a]Peripheral blood leukocytes were isolated as described in Materials and Methods. Plasma membranes were prepared by hypotonic lysis of glycerol loaded cells followed by differential centrifugation and were adjusted to a protein concentration of 100 μg/ml [27].
[b]The HLA antigens on the cell surface were taken from the literature for Daudi [28], Molt-4 [25], SB, and HSB-2 [29].
[c]PHA-M was used at 1% v/v. Cell-free CM were harvested after 72 hr of incubation at 37°C. The CM were assayed for TCGF activity as described in Materials and Methods and the units of activity were calculated by probit analysis using a highly active CM as an arbitrary standard of 1 unit/ml.

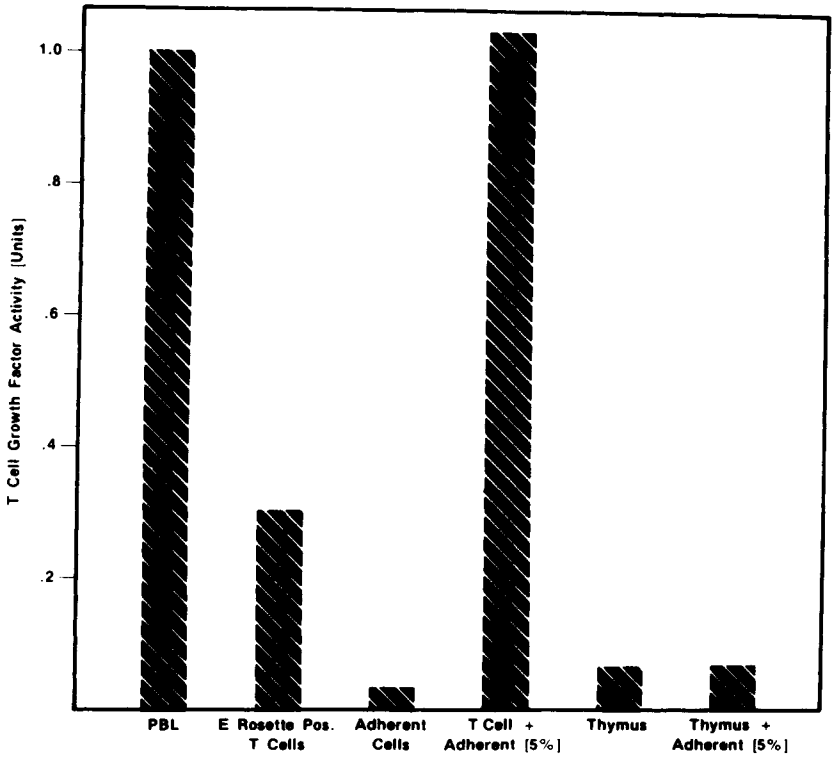

Fig. 4. Cellular requirements for TCGF production. Peripheral blood leukocytes from a single donor at 10^6 cell/ml in the presence of PHA and Daudi cells as described in Materials and Methods. Ablation of T-cells with anti-T-cell serum was complement mediated. Adherent cells were removed by nylon wool, and mature T-cells were separated by sheep erythrocyte rosetting. Experiments were done in triplicate with the standard error less than 10%.

described for murine T-cells [11], was modified for use with human T-cells (Fig. 1). To ensure specificity of the assay, the T-cells need to be in culture for at least 20 days and require the addition of TCGF for further growth. The microassay requires only 0.2 ml of sample to generate a dose-response curve, which allows several samples to be conveniently tested in one day. The use of crude Ly-CM in the assay resulted in inhibition of TCGF-directed ^{3}H-TdR incorporation at high doses of Ly-CM. The addition of PHA at the optimal mitogenic dose to the assay also inhibited some but not all cultures by 30–60%. Inhibitors, however, could be removed by fractionation of Ly-CM by ion-exchange chromatography [14], permitting the generation of sigmoid dose-response curves (Fig. 2).

The statistical methods used to determine interferon levels [24] were employed to quantitate the amount of TCGF in a given CM. A standard lot of TCGF was arbitrarily assigned a value of one unit/ml. The data from a microassay were analyzed by assigning a value of 100% to the highest value of cpm of ^{3}H-TdR incorporated and expressing all other points as a percentage of the maximum. By plotting these values against the logarithm of the media dilution on probability paper, a straight line results where the points fall within two standard deviations of the mean (50% point) (Fig. 2). The TCGF activity of an unknown sample can then be determined from the standard curve.

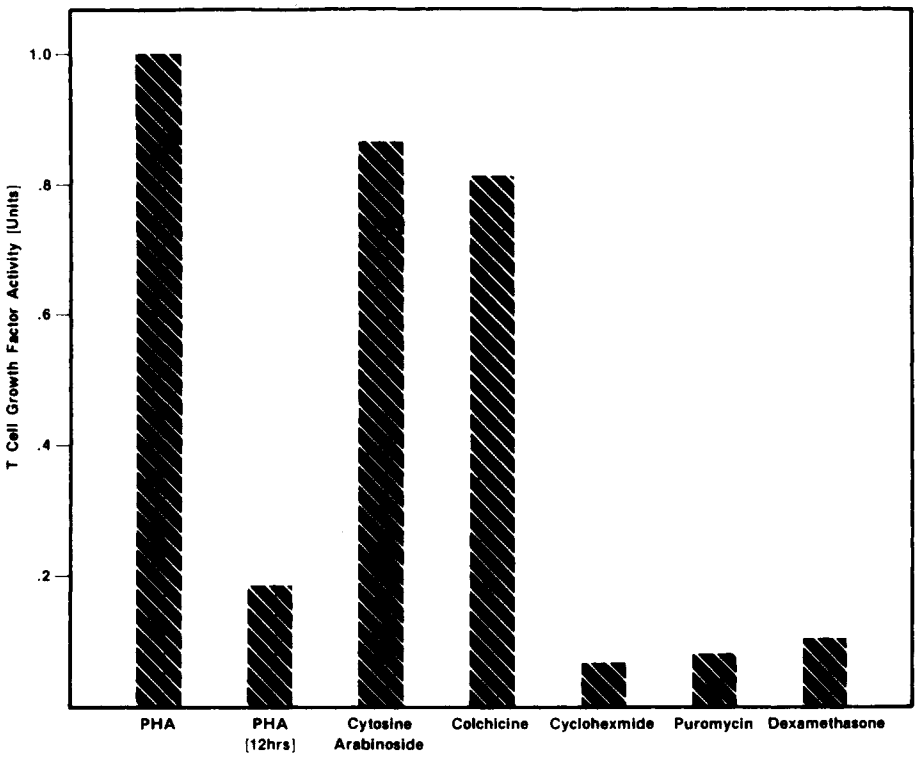

Fig. 5. The effect of macromolecular inhibitors on TCGF production. CM was prepared after 72 hr of incubation of single donor PHA-stimulated peripheral blood lymphocytes with mitomycin C-treated Daudi cells. The CM was dialyzed against 0.15 M phosphate buffered saline. Control CM, CM from cytosine arabinoside (10 μg/ml) treated cultures, CM from colchicine (100 μg/ml) treated cultures, CM from cyclohexamide (50 μg/ml) treated cultures, CM from puromycin (10 μg/ml) treated cultures, and CM from dexamethasone (10^{-6}M) treated cultures were assayed for TCGF activity. Experiments were done in triplicate with standard error less than 10%.

Production of TCGF by PHA-stimulated PBL from pooled multiple donors and from single donors was compared. Using a CM from PBL of multiple donors which was able to support the proliferation of T-cells for sixty days as the standard, we found that in all batches prepared from multiple donors' PBL gave comparable activity (Table I). In testing the PBL from 18 individual donors, most of the samples had less than 10% of the activity of the standard but some, in particular those CM harvested after 24 hr of incubation, stimulated as much as 30% of the incorporation of thymidine as standard (Table I). However, neither concentration by Amicon filtration nor fractionation by ion-exchange chromatography produced samples which were able to maintain growth of T-cells through two successive passages. We considered these samples negative, although development of a more sensitive assay, such as a radioimmune assay, may allow detection of low levels of TCGF in these CM. Use of other mitogens, removal of adherent cells, and use of different serum components did not alter these findings (Table I).

The addition of mitomycin C-treated Daudi cells to PHA-stimulated PBL from individual donors produced TCGF activity equal in amount to that produced by pooled multiple donors (Table II). This enhanced production has been observed with other lym-

phoblastoid cell lines [30]. The rationale for the use of B-cell lines was to provide a source of allogeneic stimulation and thus eliminate the need for mixing several donors. However, a recent study claimed that pooling allogeneic lymphocytes had no effect on TCGF production [31]. Whatever the basis for the effect, it is clear that not all lymphoblastoid cell lines are active (Table IV). Mixing supernatants of B-cell lines and of PHA-stimulated PBL did not increase TCGF production. This and the observation that crude plasma membrane preparations of Daudi cells could replace intact cells in production of TCGF shows that B-cell lines stimulate TCGF production rather than effect the action of TCGF.

It is possible that Ia-like antigens may be involved in the enhancing effect of lymphoblastoid cell lines in producing TCGF. Daudi cells, which stimulate TCGF production, have HLA-D antigens but no HLA-A, B, or C on their cell surface. Molt-4, a cell line which did not stimulate TCGF production, has HLA-A and B but not D antigens. It was found that over 90% of the cultured human T-cells possess HLA-DR_w (Ia-like) antigens after 8 days of culture and that these cultured T-cells also express the same HLA-DR_w phenotype as the fresh B-cells of the donor [18], suggesting that the Ia antigens were not absorbed onto the T-cells from the Ly-CM. Thus, it seems to us that Ia-like antigens play an important role in T-cell proliferation, and its relationship to TCGF is under study in our laboratory.

Mature T-cells are required for TCGF production. Depletion of E-rosette positive cells from mononuclear cells and the use of anti-T-cell sera, which is cytotoxic to T-cells, greatly reduced the production of TCGF (Fig. 4). Some evidence with murine cells indicate that the T-cell which releases TCGF belongs to a functionally distinct T-cell subset. Using specific antisera to lyse T-cell subsets, Wagner and Rollinghoff [32] found that the removal of Ly 2,3+ cells had no effect on TCGF production, whereas removal of the Ly 1+ cells completely abrogated its release. Interestingly, Ly 1+ murine T-cells have Ia antigens on their cell surface.

In addition to activation by a lectin or antigen, TCGF requires another signal derived from an adherent cell population, probably a macrophage (Fig. 4). It has been shown that in TCGF production by murine cells the adherent cell requirement can be replaced by soluble macrophage products and that TCGF production may be stimulated by macrophage-derived lymphocyte activating factor (LAF) [33, 34].

However, it is difficult to distinguish whether biologically active molecules like LAF, PHA, or Ia antigens act solely on TCGF production or also act synergistically with TCGF to amplify TCGF action. In addition, it is not known whether some TCGF producer cells survive in long-term cultures so that when these substances are put directly in the TCGF assay, the increase in ^{3}H-TdR incorporation observed is due to low level TCGF production during the assay period.

Studies on the regulation of production of TCGF with inhibitors of macromolecular synthesis showed that inhibition of DNA synthesis and cellular proliferation had no effect on TCGF release (Fig. 5). However, little or no TCGF activity was present if inhibitors of protein synthesis were used. Thus, TCGF production can occur in a non-dividing cell, probably in a terminally differentiated T-cell without the need for proliferation but requiring binding of a stimulating agent and protein synthesis. It is not clear whether the T-cell involved in production proliferates in response to TCGF or not. All the normal long-term T-cell lines that have been developed, whether they were helper, cytotoxic, or polyclonal in function, have not contained cells capable of producing enough TCGF to maintain growth of the cultures [2–10].

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