Brief Definitive Report

MITOGEN-INITIATED SYNTHESIS AND SECRETION OF T CELL GROWTH FACTOR(S) BY A T-LYMPHOMA CELL LINE*

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Spleen cells stimulated by concanavalin A (Con A) produce a factor or factors (T cell-growth factor [TCCF]), TCGF-S, that stimulates T cell replication (1–9). The production of TCGF-S involves at least two cell types, a Thy-1^+ cell and an Ia^+ cell (1, 2). The subpopulation of T cells that yields TCGF-S appears to be Lyt-$1^+2^-3^-$ (3–5, 10). It is not known if TCGF activity is the property of a single molecule or of a group of related molecules.

The results reported here derive from a search for a TCGF-producing tumor cell line. Although no cell line examined yielded TCGF spontaneously, 1 of 12 (EL-4 azg^r) T-lymphoma cell lines and, to a lesser extent, the parental EL-4 line, produced TCGF (TCGF-E) when stimulated by T mitogens; the TCGF-E produced has the molecular size and functional properties of TCGF-S.

Materials and Methods

Tumor Cell Lines. The EL-4 cell line was originally obtained from Dr. D. B. Amos, and the EL-4 azaguanine-resistant (EL-4 azg^r) cell line was established in this laboratory and maintained in the medium that contained 100 μM 8-azaguanine. These lines were Lyt-1^+, Lyt-2^-3^-, and Thy-1^+, on repeated testing with monoclonal antibody. Other T-lymphoma cell lines were obtained from the Salk Institute for Biological Studies, San Diego, Calif. and have the markers indicated in Table I.

Induction of TCGF Production. Various numbers of cells (1×10^6–4×10^6) of each T-lymphoma cell line were cultured with or without a mitogen (Con A, phytohemagglutinin [PHA-P], pokeweed mitogen [PWM], or lipopolysaccharide [LPS]) in 2 ml RPMI-1640 supplemented with 2.5% or 5% fetal calf serum (FCS), 5×10^{-5} M, 2-mercaptoethanol (2ME), 10 mM Hepes, and antibiotics in 24-well plates (Linbro Chemical Co., Hamden, Conn.). The cultures were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. The supernatant media were harvested after various durations (3–36 h) and filtered through 0.22-μm filters and stored at −20°C until use.

Assay Cells for TCGF. Culture supernates or fractions obtained by chromatography were assayed for TCGF activity primarily on a cloned C57BL/6 anti-DBA/2 cytotoxic T cell line (BAD-1, Lyt-$1^-2^+3^+$) that was developed and maintained in this laboratory (11) for >18 mo. This line is absolutely TCGF dependent and it does not proliferate in response to mitogens or antigen. In addition to this line, TCGF-E activity was assayed on a C57BL/6 anti-DBA/2 alloreactive cell line (BADCXA-2, Lyt-$1^+2^-3^-$), Con-A-stimulated T cell blasts, LPS-stimulated B cell blasts, thymocytes, and nylon-wool-column-passed spleen cells.

Assay of TCGF Activity. The method of Gillis et al. (12) was modified as follows: assay cells were washed and resuspended in RPMI-1640 with 2.5% FCS, 5×10^{-5} M 2ME, 10 mM Hepes,

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TABLE I

*Mitogen-induced TCGF Production by T-Lymphoma Cell Lines**

Cell line tested	Surface antigen markers‡			Spontaneous secretion of TCGF	Mitogen-induced TCGF activity by mitogen§			
	Thy-1.2	Lyt-1	Lyt-2		Con A (5 μg/ml)	PHA-P (1 μl/ml)	PWM (10 μl/ml)	LPS (10 μg/ml)
EL-4	+	+	−	702‖ (0.91)¶	5,294 (6.9)	869 (1.1)	898 (1.2)	1,186 (1.5)
EL-4 azg^r	+	+	−	1,021 (1.3)	17,082 (22.2)	2,600 (3.4)	1,101 (1.4)	3,779 (4.9)
BW5147	−	−	−	588 (0.88)	599 (0.78)	260 (0.34)	1,014 (1.3)	903 (1.2)
BW5147 G1.4.	−	−	−	720 (0.93)	707 (0.92)	380 (0.50)	942 (1.2)	966 (1.3)
BW5147 G1.4. Oua^r	−	−	−	688 (0.90)	719 (0.94)	309 (0.40)	851 (1.1)	766 (1.0)
T1M1.4.G.1.3	+	−	±**	633 (0.82)	564 (0.73)	326 (0.42)	677 (0.88)	859 (1.1)
S49.1G.3	+	−	−	530 (0.69)	583 (0.76)	282 (0.37)	590 (0.77)	743 (0.97)
S49 Thy-1$^-$	−	−	−	436 (0.57)	437 (0.57)	246 (0.32)	480 (0.63)	520 (0.68)
R 1.1.	+	−	−	689 (0.90)	732 (0.94)	351 (0.46)	754 (0.98)	746 (0.97)
R 1.G1.	+	−	−	713 (0.93)	696 (0.91)	425 (0.55)	769 (1.0)	859 (1.2)
WEHI-7	+	−	−	497 (0.64)	784 (1.0)	476 (0.62)	734 (0.96)	818 (1.1)
WEHI-22.1	+	−	−	604 (0.79)	655 (0.85)	373 (0.49)	654 (0.85)	787 (1.0)

* In this experiment, control (medium) incorporation was 768 ± 61 cpm and 7- or 28-times-concentrated EL-4 azg^r supernates were 854 ± 113 or 1,101 ± 15 cpm, respectively. Cell extracts of EL-4 azg^r made by freeze-thawing three times gave 758 ± 49 cpm; TCGF-S at 25% concentration gave 10,192 ± 635 cpm and at 12% gave 7,775 ± 1,395; purified TCGF-S at 25% concentration gave 22,006 ± 149 cpm. TCGF-E obtained from irradiated EL-4 azg^r (5,000 or 7,000 rad) plus Con A (5 μg/ml) gave 16,832 ± 1,013 and 16,901 ± 1,333, respectively.

‡ EL-4 and EL-azg^r were T-200$^+$. All lines are sIg$^-$. Thy-1, Lyt, and T-200 were determined by indirect immunofluorescence assay with monoclonal antibody, and sIg by direct immunofluorescence.

§ 1 × 10^6 cells/ml of each cell line were cultured with or without a mitogen in 2 ml of 2.5%-FCS culture medium in 24-well plates. After 18 h of culture, supernates were assayed for TCGF activity on cell line BAD-1. [^{3}H]thymidine incorporation was measured for the last 6 h of the 28-h incubation.

‖ [^{3}H]thymidine incorporation (mean cpm).

¶ Stimulation index; mean [^{3}H]thymidine incorporation of experiment/control.

** Minimal but detectable binding of anti-Lyt-2 monoclonal antibody.

and antibiotics. 5 × 10^4 cells in 100 μl medium were placed in 96-well round-bottomed microplate (Linbro Chemical Co.) followed by 100 μl of TCGF sample. Microplates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After 22, 24, or 48 h of culture, 0.5 μCi of [^{3}H]thymidine (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.; 1.9 Ci/mM sp act) was added to each microplate well and cultured additional 6 or 24 h. [^{3}H]Thymidine incorporation was determined by scintillation counting.

To assay for enhancement by TCGF of mitogen-induced responses in thymocytes or spleen T cell subpopulations, 5 × 10^5 cells in RPMI-1640 supplemented with 0.5% mouse serum, 2ME, Hepes, and antibiotics, in 100 μl were distributed in 96-well Microtest II plates (Linbro Chemical Co.); 100 μl of 0–25% purified TCGF-E was added, with or without 0.3 μg/ml of Con A. After 24 h of culture, 0.5 μCi of [^{3}H]thymidine was added; the cultures were harvested 24 h later.

Purification Procedures. Purification of TCGF-E was accomplished by Sephadex G-100 chromatography (100- × 2.6-cm column; pH 7.4; phosphate-buffered saline (PBS) eluent; constant flow elution of 15 ml/h) of various culture supernates after 50- to 70-fold concentration (Diaflo ultrafilter PM10; Amicon Corp., Lexington, Mass.). Standards for molecular weight estimation included ^{125}I-bovine serum albumin, ^{125}I-Rauscher virus P30 (kindly supplied by Dr. Charles Simrell and Dr. Paul Klein), ^{125}I-cytochrome *c*, and [^{3}H]-Con A (Radiochemical Centre, Amersham, England).

Assay for Cytotoxicity. Cytotoxic activity was assessed by a ^{51}Cr-release assay system described in detail elsewhere (13).

Results

TCGF Production by Murine T-Lymphoma Cell Lines. 12 cultured cell lines of T-lymphoma origin were investigated for spontaneous or induced production of TCGF under indentical conditions as shown in Table I. None of the 12 T-lymphoma lines produced TCGF spontaneously. Even 7- to 28-times-concentrated supernates and cell cytoplasm extracts of EL-4 azg^r contained no detectable TCGF activity. In contrast

to the 10 other T-cell lines, both EL-4 and EL-4 azg^r secreted significant amounts of TCGF into culture supernates when stimulated by mitogens. Con A was most effective of all mitogens in inducing TCGF production by EL-4 and EL-4 azg^r. Production of TCGF-E was rapidly induced, detectable at 6 h, and maximal at 18–24 h. The dose-response, time-course, and cell-density kinetics are illustrated in Fig. 1. Irradiation (5,000–7,000 rad) did not affect TCGF production by Con A-stimulated EL-4 azg^r. LPS, a putative B cell mitogen, consistently stimulated low but significant production of TCGF-E in the EL-4 lines. This unexpected observation is still being investigated in light of previous studies suggesting that LPS might have some role in stimulating thymus cells (14).

Characterization of TCGF-E Obtained from Con A-stimulated EL-4 azg^r. Supernates from both Con A-stimulated EL-4 azg^r and Con A-stimulated murine spleen cells were concentrated 50- to 70-fold and fractionated on Sephadex G-100: Each fraction was assayed for [^{3}H]Con A and for functional TCGF activity on a cloned cytotoxic T cell line or on Con A blast cells. As shown in Fig. 2, TCGF-S and TCGF-E had identical peak activity in the fractions that contained the P30 marker, indicating a molecular size on the order of 30,000 dalton, a size range clearly separable from that of [^{3}H]Con A. This partially purified product is termed PTCGF-E and was used in studies reported below.

Comparison of Various Biological Activities of TCGF-S and TCGF-E. TCGF-E has been tested for each functional effect described for TCGF-S (1, 7–9). Nylon-wool-column-passed C57BL/6 spleen T cells were very slightly stimulated or unaffected by PTCGF-E alone, but responded strongly to suboptimal concentrations of Con A (0.3 μg/ml) when very dilute PTCGF-E was added. Thymocytes responded minimally to Con A or to PTCGF-E alone, but proliferated in the presence of both as shown in Table II. LPS blasts generated from either C57BL/6 or C57BL/6 nu/nu failed to proliferate with PTCGF-E. The growth supporting activity of PTCGF-E was generally equivalent upon DBA/2-, AKR-, or A/J-derived T cells, indicating no strain specificity. In experiments not shown here, the PTCGF-E requirement for generating cytotoxic

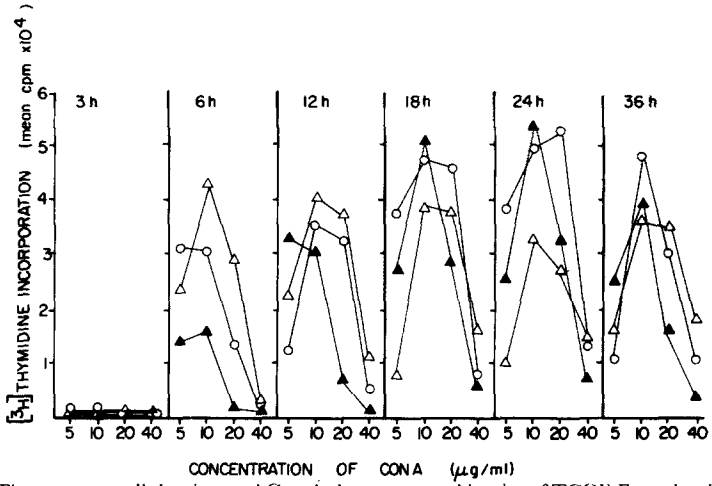

FIG. 1. Time-course, cell density, and Con A-dose-response kinetics of TCGF-E production by EL-4 azg^r. EL-4 azg^r cells (5 × 10^5 cells/ml [▲]; 1 × 10^6 cells/ml [○]; 2 × 10^6 cells/ml [△]) were cultured with 5, 10, 20, or 40 μg/ml of Con A for various intervals in 5%-FCS culture medium. Supernates were assayed for TCGF activity on cell line BAD-1. [^{3}H]Thymidine incorporation was measured for the last 24 h of the 48-h incubation.

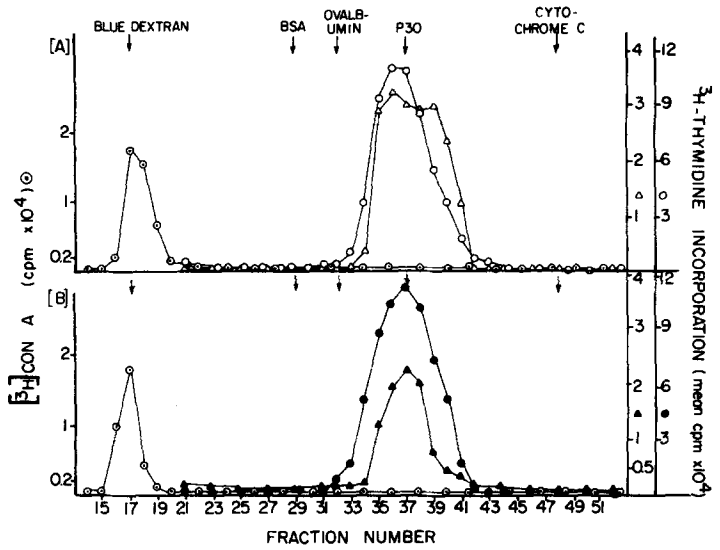

FIG. 2. Fractionation of TCGF-E (A) or TCGF-S (B) by Sephadex G-100 chromatography. Concentrated TCGF-E or TCGF-S was fractionated as described in Materials and Methods and each fraction was assayed for TCGF activity on cell line BAD-1 (△ or ▲) or Con A blasts (○ or ●). [^{3}H]Thymidine incorporation was measured for the last 24 h of the 72 h incubation. [^{3}H]Con A (◉) was added to the TCGF before fractionation. The peaks shown represent 22% of the counts added to the column; 78% was bound to Sephadex G-100 at the application point.

TABLE II

PTCGF-E Effect on Various Kinds of T-Cell Blasts, B Cell Blasts, Normal Spleen T Cells, or Thymocytes

Cultured cells and conditions	[^{3}H]Thymidine incorporation: percent PTCGF-E:			
	0	6	12	25
	mean cpm ± SD			
T or B cell blasts*				
BAD-1 CTL	214 ± 88	12,761 ± 1,923	36,205 ± 4,172	52,661 ± 4,464
BADCXA-2 alloreactive T cell	478 ± 112	54,564 ± 711	55,750 ± 928	53,129 ± 1,722
C57BL/6 Con A blast	504 ± 134	32,050 ± 1,849	49,843 ± 1,741	60,740 ± 1,410
C57BL/6 LPS blast	8,782 ± 584	10,070 ± 250	11,151 ± 200	10,437 ± 489
C57BL/6 nude LPS blast	9,373 ± 597	12,241 ± 877	12,401 ± 70	12,392 ± 329
DBA/2 Con A blast	1,395 ± 153	ND	39,780 ± 1,283	46,806 ± 1,000
AKR Con A blast	892 ± 135	ND	19,232 ± 1,166	33,378 ± 1,917
A/J Con A blast	936 ± 247	ND	18,955 ± 761	28,965 ± 1,000
Spleen cells and thymocytes‡				
C57BL/6 spleen T cell	146 ± 11	656 ± 42	1,409 ± 30	2,606 ± 329
C57BL/6 spleen T cell + Con A (0.3 μg/ml)	10,978 ± 2,284	32,371 ± 2,666	57,228 ± 3,718	94,702 ± 1,200
C57BL/6 thymocyte	158 ± 42	2,245 ± 403	2,572 ± 172	3,548 ± 287
C57BL/6 thymocyte + Con A (0.3 μg/ml)	2,023 ± 510	23,461 ± 2,624	28,854 ± 325	35,773 ± 3,052

After 24 h of culture, 0.5 μCi of [^{3}H]thymidine was added; the cultures were harvested 24 h later. The results were expressed as mean cpm ± SD in triplicate cultures. ND, not determined.

* 5×10^4 cells of each T cell blast were cultured with or without indicated concentrations of PTCGF-E.

‡ 5×10^5 cells of nylon-wool-column-passed spleen cells or thymocytes were cultured with indicated concentrations of Con A and/or PTCGF-E.

activity against P815 target cells in C57BL/6 anti-DBA/2 mixed lymphocyte cultures was assayed on cells collected after 10 d or after a sixth restimulation in a long-term culture. Alloreactive cells alone had little residual cytotoxic activity 15 d after

restimulation. 30% PTCGF-E augmented cytotoxic activity to a level even greater than that induced by specific stimulator cells.

Discussion

Evidence is presented that a variant of the EL-4 cell line (EL-4 azg^r) and, to a lesser extent, the parental EL-4 line produced TCGF when stimulated by Con A. The producer lines carry the Lyt-1 marker, whereas all nonproducer T cell lines examined did not. Until more lines with Lyt-1 markers are examined, however, it can be concluded only that this phenotype identifies a TCGF-producer cell. However, it is consistent with the data of others that suggest that TCGF production is a function of Lyt-1-bearing T cells (3–5).

Kinetic studies showed most TCGF-E production occurred early (6 h) after stimulation by Con A. Production of TCGF-E was radiation resistant. Biochemical properties and biological activity suggest strongly the indentity of TCGF-E and TCGF-S, including: similar molecular weights, identical effects upon T cell mitogen responses, T cell blast proliferation, generation of cytotoxic T cells, absence of effects upon normal resting T cells, and lack of strain specificity.

Mitogen induction for TCGF production by EL-4 azg^r appeared to be an absolute requirement. EL-4 azg^r produced no detectable TCGF-E intracellularly or extracellularly, unless stimulated with Con A. This suggests that TCGF production is a differentiated function of the cell line, requiring only a specific signal to initiate synthesis and secretion of the product or products assayed as TCGF. In this case, the triggering effect is supplied by a lectin that binds to the cell surface without any accessory cell requirement. This signal can also be supplied by binding of xenogeneic anti-Thy-1 sera to the membrane of EL-4 azg^r (S. Shimizu and R. T. Smith. Unpublished data.). Detectable low level secretion of TCGF was stimulated by PHA-P and LPS.

An FcR$^+$ (6, 15) or an Ia$^+$ (1, 2) accessory cell is required to induce TCGF production by anti-Thy-1 antibody or Con A, respectively. Therefore, the EL-4 azg^r cell line appears to bypass the requirement for any signal provided by an accessory cell. If the accessory cell requirement is, as has been proposed (6), related to its role in augmenting translateral aggregation by mitogen-bound glycoproteins in the T cell membrane, then the T-lymphoma line EL-4 azg^r has acquired a capacity to accomplish this unaided. That LPS can also stimulate minimal TCGF production in EL-4 azg^r suggests that the bypassed accessory cell mechanism yields a less discriminating responsiveness than normal T cells in terms of T cell specificity of the mitogenic stimulus.

These observations suggest a practical source of TCGF for use in further investigation of mechanisms of T cell activation, and a more homogeneous source of TCGF for characterization of the molecule or molecules involved. Moreover, they provide a model cell in which to examine the signal mechanism by which membrane glycoprotein binding can activate TCGF synthesis and secretion.

Summary

Of 12 T-lymphoma cell lines investigated, one line, EL-4 azg^r (Thy-1$^+$, Lyt-1$^+$, Lyt-2$^-$3$^-$, Ia$^-$, T-200$^+$, sIg$^-$, and FcR$^-$) and, to a lesser extent, the parental cell line, EL-4, produced T cell growth factor(s) (TCGF) when stimulated by the T-cell mitogen concanavalin A (Con A). Induced production of TCGF-E was detected by 6 h and maximal at 18–24 h. Purified TCGF-E from this source had an ~30,000 mol wt and

the biological activity of TCGF produced by whole spleen cells, including: augmentation of T cell-mitogen responses, cytotoxic T lymphocyte (CTL) proliferation support dependence, augmented generation of CTL, lack of strain specificity, and failure to stimulate resting T cells. TCGF-E is neither synthesized or secreted by this lymphoma cell line unless stimulated by Con A. X-irradiation up to 7,000 rad failed to inhibit synthesis and secretion. These observations have a practical application in providing a relatively homogeneous clonal cell product for T cell culture support and for structural and functional studies of the TCGF molecule(s). They suggest also a model for examining mechanisms of triggering production and secretion of a regulatory molecule that controls T cell functions.

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