HUMAN CUTANEOUS T CELL LYMPHOMA AND LEUKEMIA CELL LINES PRODUCE AND RESPOND TO T CELL GROWTH FACTOR

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The proliferation of normal human T lymphocytes involves an initial activation by antigen or lectin followed by a growth stimulus provided by a lymphokine-designated T cell growth factor (TCGF).[1] TCGF is present in the conditioned media (CM) of mitogen-stimulated human mononuclear cells and is probably released by a subset of T lymphocytes. The addition of TCGF to activated T cells allows their growth in liquid suspension culture for long periods (1, 2). This culture system is now widely used, and has been extended to several animals as well as to man (for recent reviews see 3, 4).

Recent studies from our laboratory (5) and others' (6–8) have clearly shown that TCGF purified free of antigen or lectin, and not the antigen or lectin, supports the growth of T cells. The role of the antigen in this process appears to be both to stimulate the TCGF-producer cell to produce TCGF and to activate the TCGF-responder cell so that it becomes capable of binding TCGF (5–8). Purification and characterization of human TCGF obtained from the CM of phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells pooled from several healthy donors (5) has shown that this molecule is a small protein (~13,000 mol wt) that can aggregate to larger molecular weight forms,[2] and is the sole requirement for long-term growth of activated T cells. Other recent results suggest that at least some forms of the protein are glycosylated (9).

In contrast to normal T cells, which demonstrate an absolute requirement for prior lectin or antigen stimulation for response to TCGF, neoplastic cells of mature T cell origin (sheep erythrocyte receptor positive, terminal deoxynucleotidyl transferase [TdT] negative) respond directly to lectin-free partially purified TCGF (10). Through exploitation of this property, TCGF-dependent cell lines expressing phenotypic char-

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[1] *Abbreviations used in this paper:* BSS, basic salt solution; CM, conditioned media; CSA, colony-stimulating activity; CTCL, cutaneous T cell lymphoma-leukemia cells; FCS, fetal calf serum; IM, infectious mononeucleosis; PBL, peripheral blood leukocytes; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; PMSF, phenylmethosulfofluoride; TCGF, T cell growth factor (L-TCGF, lymphoma-leukemia TCGF; N-TCGF, normal TCGF; ppTCGF, partially purified TCGF); TLCK, Nα-*p*-tosyl-lysinechloromethyl ketone; TdT, terminal deoxynucleotidyl transferase; TPCK, 1-tosylamino-2-phenylethylchloromethyl ketone; ppTCGF, partially purified TCGF.

[2] Mier, J. W., and R. C. Gallo. Purification and some properties of human T cell growth factor. Submitted for publication.

acteristics consistent with the tumor cells of origin have been derived from several samples of cutaneous T cell lymphomas and leukemias (CTCL) (10, 11) and T cell acute lymphoid leukemias (10). Two of these CTCL cell lines, HUT-102 and CTCL-2, initially dependent on TCGF for growth, have lost their requirement for exogenous TCGF after serial passage. The reason for this transformation is unknown, but the autonomous growth of these cells may be due to constitutive production of TCGF or a related molecule eliminating the need for exogenous TCGF.

In this paper we report the following observations: (*a*) TCGF is constitutively produced by three human neoplastic CTCL lines and this activity is found in the conditioned media and on the cell surface of these lines; (*b*) TCGF activity is adsorbed by these lines and not by other human tumor cell lines; and (*c*) the rate of proliferation of these cell lines can be increased by the addition of TCGF. These data suggest that TCGF or a related molecule is an essential component of the continuous proliferation of these transformed T cell lines. It is possible that a major abnormality in these disorders is the capacity of the same malignant cell to produce and respond to its own growth factor. Since established cell lines of neoplastic mature T cells that produce their own TCGF and grow independent of exogenous TCGF are unusual (3 lines of >30 attempts), an alternative interpretation, and one more consistent with the results, is that production and response to TCGF by the same cell ("autostimulation") is an unusual variant of some transformed mature T cells that is not essential to their neoplastic conversion but favors their selective growth as established cell lines.

Materials and Methods

Preparation of TCGF from Normal Human Peripheral Blood Leukocytes. Heparinized whole human blood from normal donors mixed with plasmagel (0.3 ml/ml blood) was incubated without agitation at 37°C for 1 h. The leukocyte-rich plasma was collected and passed through nylon fiber filled columns (Dupont type 200 nylon, DuPont Instruments, Wilmington, Del.). The cell pellet resuspended in culture media was mixed with 10–20 similarly prepared cell pellets from ABO-compatible donors. CM containing TCGF was prepared by incubating 10^6 leukocytes/ml in tissue culture media containing 0.25% bovine serum albumin and 1% PHA-M (Difco Laboratories, Detroit, Mich.) at 37°C for 72 h (12, 13). The cell-free CM was then collected, filtered under sterile conditions, and stored at −20°C. Partially purifed TCGF was prepared by precipitation with $(NH_4)_2SO_4$ and fractionation on a DEAE-Sephrose column as previously described (5). This material was sterilized and stored at 4°C before use.

Cultured T Cells. Long-term cultures of human T cells were maintained as previously described (1, 2). These cultures were resuspended at 5×10^5 cells/ml in crude CM containing TCGF (50% vol:vol). After 4–5 d, the cells reached a saturation density of $1–2 \times 10^6$ cells/ml and were diluted to 5×10^5 cells/ml by the addition of fresh TCGF-containing media. This process was repeated every 4–5 d.

TCGF Assay. To estimate the amount of TCGF present in any sample, a microassay was used as previously described (13, 14). Normal T cells initially activated with PHA were cultured for 20 d in the presence of added TCGF. These cells can not proliferate without TCGF and can be used as target cells in the TCGF assay. Such cells, kept at their saturation density for 24 h, were washed free of TCGF, resuspended in media containing 15% fetal calf serum (FCS), and placed in 96-well microplates (no. 3596, Costar, Data Packaging, Cambridge, Mass.) at 2×10^4 cells/well. An equal volume of a serial dilution of the sample to be assayed was then added. After incubation for 48 h, 0.5 μCi of [^{3}H]TdR (specific activity 0.36 Ci/mM, Schwartz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) were added to each well. After incubation for an additional 16 h, cultures were harvested on glass fiber filter strips for determination of [^{3}H]TdR incorporation. The amount of TCGF is estimated from an analysis of the dose-response kinetics of serial dilutions of the test sample (13, 14). Briefly, a standard TCGF preparation is titrated in each experiment to determine the dilution that gives the maximum

counts per minute (cpm). Further dilution points are expressed as a percentage of the maximum cpm and the TCGF activity of each test sample is then determined from a regression line generated by serial dilutions of the sample.

Cell Lines. All TCGF-independent cell lines including HUT-102, HUT-78, and CTCL-2, cell lines derived from CTCL samples (11, 12), were grown in RPMI 1640 (Grand Island Biological Company, Grand Island, N. Y.) containing 10% heat-inactivated FCS (Biofluids, Rockville, Md.). HUT-102 and CTCL-2 initially required TCGF for growth; HUT-78 initially required concanavalin A for growth. However, after serial passage, growth of these cells became independent of these factors. The lymphoblastoid cell lines, Daudi, HSB-2, CCRF-CEM, EB-3, and RPMI 8866 were received from The American Type Culture Collection (Rockville, Md.). All other lymphoblastoid were a gift from Dr. J. Minowada (Buffalo, N. Y.). The TCGF-dependent CTCL cell lines were grown using partially purified TCGF as previously described (10), otherwise the conditions were the same as for the TCGF-independent cell lines.

TCGF Production from Human Tumor Cell Lines. Various concentrations of PHA-P (Difco Laboratories, Detroit, Mich.) with and without addition of phorbol myristate acetate (PMA) (10 ng/ml) were added to these cell lines cultured at cell concentrations of 10^6 and 10^7 cells/ml. CM were harvested at 24-, 48-, and 72-h intervals, concentrated by precipitation with $(NH_4)_2SO_4$ (5), and assayed for TCGF activity as described above.

Acid Glycine Elution of TCGF. Elution of cell surface proteins of cells was accomplished using published procedures (15). In brief, cells were washed in Dulbecco's phosphate-buffered saline (PBS), centrifuged at 1,800 rpm for 15 min, and the resultant cell pellet resuspended in 0.2 M glycine, pH 2.8, at a cell density of 2×10^8 cells/ml. After incubation at 4°C for 15 min, the cells were removed by centrifugation, and the supernate titrated to pH 7 by addition of solid Tris base. The supernate was then concentrated 10–20-fold by precipitation with $(NH_4)_2SO_4$ as previously described (5).

Response of CTCL Lines to Exogenous TCGF. Cells were incubated in media containing the desired concentration of TCGF or media alone at 37°C. Media was replenished daily. 1-ml aliquots were incubated daily with 0.5 μCi/ml of [^{3}H]TdR for 18 h and the [^{3}H]TdR incorporation was used to determine a stimulation index of:

$$\frac{\text{cpm } [^3\text{H}]\text{TdR incorporation with TCGF}}{\text{cpm } [^3\text{H}]\text{TdR incorporation without TCGF}}$$

Cell counts were done by the trypan blue exclusion method and sheep erythrocyte (E) rosette determination was done by standard methods (16).

Adsorption of TCGF. All procedures were performed at 4°C. Cells were washed twice with PBS and incubated for varying lengths of time as defined in Results at several cell concentrations with partially purifed TCGF diluted to yield 50% of its maximum thymidine incorporation. This diluted sample was tested to ensure that experimental loss of TCGF would fall on the linear portion of the sigmoid dose-response curve (14). After the incubation period, the cellular material was removed by centrifugation and serial dilutions of the resulting supernates were tested for TCGF activity in a microassay. Regression lines were drawn from the data points and the fraction of TCGF activity remaining after incubation was calculated as shown in Fig. 1. In this example, a regression line was calculated from data points generated by serial $\log_2$ dilutions of an unadsorbed sample of partially purified TCGF (ppTCGF) and a second line derived from data of the identical ppTCGF preparation adsorbed with 2×10^7 CTCL-2 cells for 2 h. The slope of the line of adsorbed material divided by the slope of the line of unadsorbed material equals the TCGF remaining (30% in this case).

Adsorptions using cell membranes prepared as described below were performed under identical conditions. The membranes were removed by centrifugation at 25,000 rpm for 20 min. Other adsorptions were performed in the presence of 0.1 mM phenylmethosulfofluoride (PMSF), 0.1% sodium azide, or a mixture of protease inhibitors (PMSF, 1% trasylol, 1 mM 1-tosylamido-2-phenylethylchloromethyl ketone [TPCK] and 1 mM Nα-*p*-tosyl-lysinechloromethyl ketone [TLCK]). In these cases the cells were pretreated with the inhibitor at 37°C for 30 min the adsorptions were performed at 4°C in the presence of the inhibitor(s), and the absorbed CM were dialyzed against PBS for 72 h before assaying for TCGF. Using previously published methods (17) to assay for granulocyte-macrophage colony-stimulating activity (CSA),

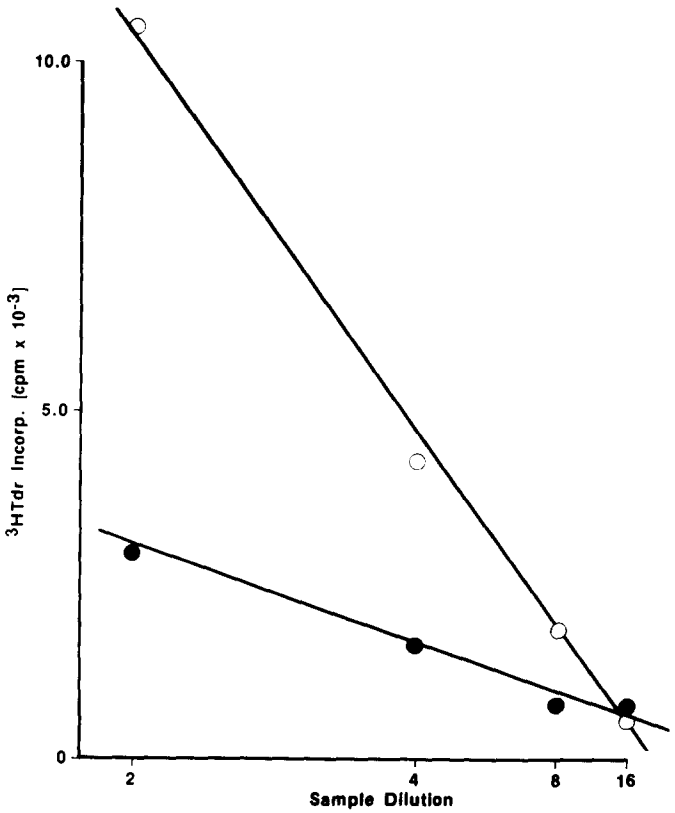

FIG. 1. Analysis of TCGF adsorption experiments. A standard preparation of partially purified TCGF was diluted to a concentration of TCGF which stimulated [^{3}H]TdR incorporation in a linear manner when serially diluted. TCGF was measured in a microassay as described in Materials and Methods. Linear regression curves were drawn for serial dilutions for both control (○) and experimental (●) data. The TCGF activity remaining in the "adsorbed" media was determined as described in Materials and Methods. CTCL-2 at 2 × 10^7 cells/ml was incubated with TCGF for 2 h at 4°C.

adsorptions with CSA were carried out to serve as a control for the specificity of the TCGF adsorption. The source of CSA was human placental conditioned media made under serum-free conditions.[3]

Preparation of Plasma Membranes. The method used was adapted from one described for cell fractionation of Raji cells (18). Cells previously incubated for 30 min in 1 mM chloroquine (to prevent lysosomal fusion) suspended at 5 × 10^8/ml in Earle's basic salt solution (BSS), pH 8.0, were slowly mixed with sufficient 90% glycerol in BSS at 37°C over a 20-min interval to yield a 40% solution. All subsequent steps were performed at 4°C. The cells were then pelleted, resuspended in Tris-HCl buffer, pH 7.4, containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$, and disrupted using a Dounce apparatus. The material was centrifuged at 1,000 *g* for 10 min and the pellet was disrupted and centrifuged again. The combined supernates (S100) were layered over a 38% sucrose cushion and centrifuged at 25,000 rpm for 2 h using a SW27 rotor in a L5-50 Ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.). The material at the interface was harvested, diluted to <12% sucrose with Tris-HCl buffer, pH 7.4, and centrifuged at 25,000 rpm for 20 min. These fractions were assayed for thymidine 5′-phosphodiesterase (19), a plasma

[3] Ruscetti, F. W., J. Y. Chou, and R. C. Gallo. 1981. Human trophoblasts: cellular source of CSA in human placental conditioned media. *Blood.* In press.

membrane marker, β-glucuronidase (20), a lysosomal marker, succinate dehydrogenase (21), a mitochondrial marker, and glucose-6 phosphatase (22), a microsomal marker.

Results

Adsorption of TCGF by Activated Normal T Lymphocytes. Incubation of a standard preparation of TCGF with peripheral blood leukocytes (PBL) previously stimulated with PHA for 5 or 7 d removed TCGF activity, and this result was directly related to the cell concentration (Fig. 2). Removal of TCGF was also time dependent; some adsorption occurred within 10 min and complete adsorption occurred after 2 h. Incubation at 4°C for 2 h with 6×10^7 PBL stimulated for 7 d removed all the activity. Cells grown with ppTCGF retain their ability to adsorb TCGF after growth in culture for 20 or 90 d (Fig. 2). Four 6×10^7 cultured T cells per ml incubated at 4°C for 2 h removed all the TCGF activity. Alloantigen-activated cultured T cells absorbed as well as lectin-activated T cells (Table I). Removal of TCGF activity by cultured T cells was not affected by the addition of 0.1 mM PMSF or 0.1% sodium azide in the incubation mixture (Table I). Incubation of the adsorption mixture at 37°C reduced the time necessary to achieve a specified reduction in activity (data not shown).

Lack of Adsorption of TCGF by Nonactivated Normal T Lymphocytes. In contrast to the

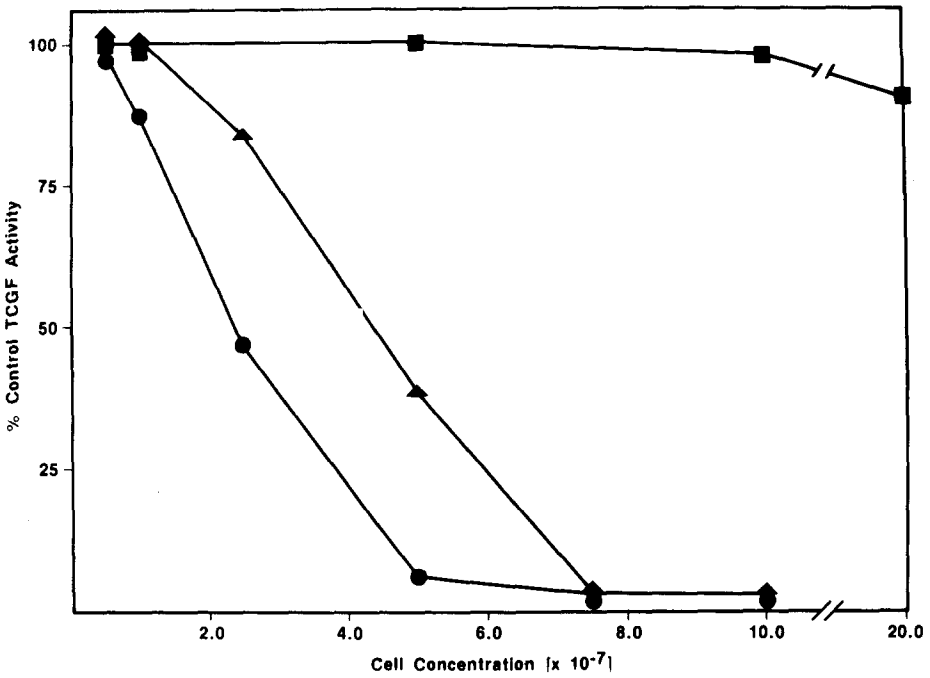

FIG. 2. Comparison of adsorption of TCGF by various normal human T cells. Incubations containing different cell populations at different concentrations were carried out with a standard preparation of TCGF at 4°C for 4 h. Cells used were the following: unstimulated normal human PBL (■); the same PBL stimulated by PHA for 7 d (▲); and cultured normal human T cells grown in the presence of TCGF for 14 d (●). The cells were first washed to remove residual TCGF before being used in TCGF adsorption assays. The TCGF activity remaining after incubation was determined as described in Fig. 1.

TABLE I

Effect of Various Conditions on the Ability of Normal T Cells to Adsorb TCGF Activity

Activation of T cells*	Additions to adsorption media‡	Number of cells (used to adsorb)	TCGF removed§
			%
PHA + TCGF	None	5×10^7	91
PHA + TCGF	PMSF	5×10^7	97
PHA + TCGF	NA azide‖	5×10^7	99
Alloantigen	None	5×10^7	86
In vivo by IM¶	None	5×10^7	93
NA	PMSF	10^8	11
NA	None	10^8	5

* Mononuclear cells isolated from peripheral blood were stimulated with PHA-P (1 μg/ml) or 10^8 mitomycin C-treated cells/ml from histoincompatible donors for 5 d and grown in partially purified TCGF (10% vol:vol). All cells were in culture for at least 3 wk before use. T cells isolated from a patient with infectious mononucleosis and unstimulated cells were adsorbed without prior culture.

‡ The adsorptions using PMSF and sodium azide were carried out as described in Materials and Methods.

§ All adsorptions were performed at 4°C for 2 h. The standard preparation of TCGF stimulated [^{3}H]TdR incorporation of 18–25,000 cpm at maximum. The TCGF rem'ved was determined as described in Materials and Methods.

‖ NA indicates cells not activated.

¶ Cells used in this experiment were mononuclear cells from a patient in an active phase of the disease.

results in the last section, incubation of a standard preparation of TCGF at 4°C for 4 h with 10^8 freshly isolated unstimulated PBL per ml removed only 4% of the TCGF activity (Fig. 2). At cell concentrations to 2×10^8/ml and 5×10^8/ml, the amount of TCGF activity removed increased to 9 and 18%, respectively. In these experiments, adsorption of TCGF was always carried out using T cells activated in vitro by conventional methods. It was of interest to know whether similar adsorption would occur with T cells activated in vivo by natural defense mechanisms. Therefore the ability of freshly isolated T cells from a patient with infectious mononucleosis (IM) to adsorb TCGF was also studied. These T cells were able to adsorb TCGF in a manner similar to that of cultured T cells without requiring in vitro activation (Table I).

Adsorption of TCGF by CTCL but Not by Other Human Leukemic Cell Types. The standard preparation of TCGF was incubated with the following human lymphoblast cell lines: SC-1, B cell in origin, NALM-1, pre-T pre-B cell, CCRF-CEM, immature T cell, HUT-102, CTCL-2, and HUT-78, three lines of mature neoplastic T cells referred to as CTCL. The number of cells used ranged from 5×10^6 to 10^8 cells/ml. HUT-102 removed TCGF activity in a cell concentration- (Fig. 3) and time-dependent manner. Adsorption occurred with as few as 5×10^6 cells. This adsorption was not affected by the presence of PMSF or sodium azide in the incubation mixture. Incubation with 2×10^7 HUT-102 cells at 4°C for 2 h removed all activity. None of the other cell lines removed >7% of the activity even when 10^8 cells were incubated for 4 h (Fig. 3). Several other human lymphoblast cell lines were assayed for their ability to adsorb TCGF by incubating 10^8 cells for 4 h at 4°C with TCGF (Table II).

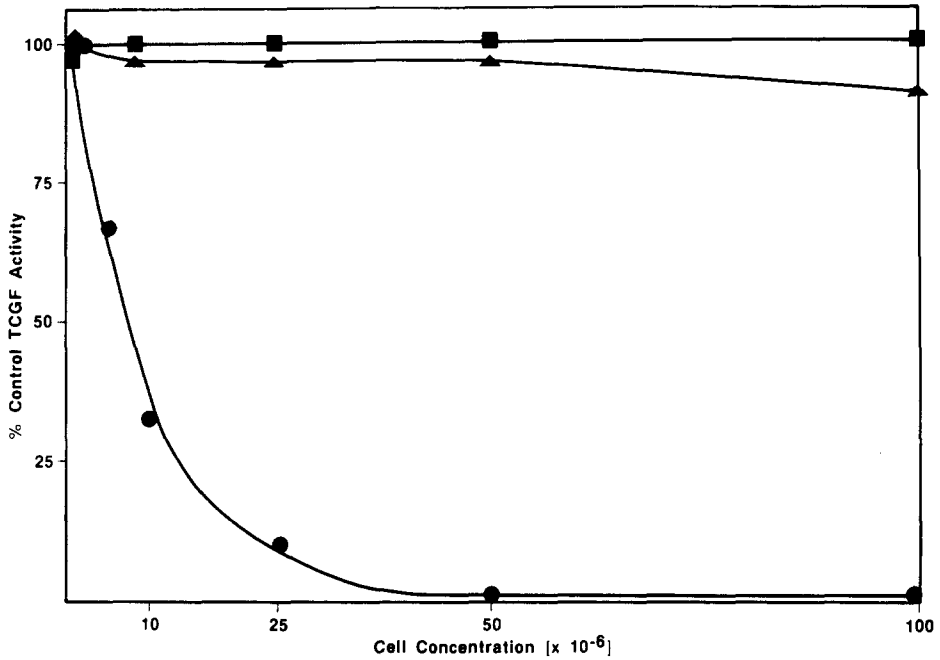

FIG. 3. Comparison of adsorption of TCGF by human neoplastic lymphoid cell lines. Incubation with different cell lines (grown with RPMI 1640 with 10% FCS) at different concentrations was carried out with a standard preparation of TCGF at 4°C for 4 h. Cells used were HUT-102 (●), SC-1 (■), and CCRF-CEM (▲). The TCGF remaining after incubation was determined as described in Fig. 1.

Only the two other CTCL cell lines, HUT-78 and CTCL-2, removed significant amounts of TCGF. When HUT-102 was incubated with a mixture of TCGF and granulocyte-macrophage CSA, the TCGF activity was completely removed, but there was no significant removal of the CSA.

Cell membranes prepared from HUT-102 cells previously incubated with chloroquine were similarly active in removing TCGF activity but not CSA activity (Table III). Adsorptions using cell membrane preparations performed in the presence of a mixture of protease inhibitors (PMSF, TLCK, TPCK, and trasylol) also resulted in complete removal of TCGF activity (Table III).

Constitutive TCGF Production by Some Human T Cell Lymphoma-Leukemia Cell Lines. Conditioned media from a variety of human tumor cell lines grown to saturation density were collected, concentrated by precipitation with $(NH_4)_2SO_4$, and tested for TCGF activity (Table IV). Of the cell lines tested in this manner, only CM from the three CTCL lines stimulated [^{3}H]TdR incorporation in a microassay for TCGF (Table IV). Attempts to induce the cell lines not constitutively releasing TCGF to produce TCGF, or to induce the three CTCL lines to make appreciably more TCGF by exposure to PHA and/or PMA, were unsuccessful. Medium supplemented with a 20-fold $(NH_4)_2SO_4$ concentrate of HUT-102 CM was able to support the growth of T cells in long-term culture (Fig. 4). The TCGF released by the lymphoma-leukemia T cells is termed L-TCGF to distinguish it from TCGF released by normal T cells now referred to as N-TCGF.

TABLE II

Comparison of TCGF Adsorption by Various Human Cell Lines

Cell lines	Origin*	Cell type‡	Number of cells (used to adsorb)	TCGF removed§
				%
CCRF-CEM	ALL	Immature T	10^8	3
HSB-2	ALL	Immature T	10^8	7
Daudi	BL	Neoplastic B	10^8	5
SC-1	—	Normal B	10^8	0
U937	HL	Monocyte	10^8	7
Nalm-1	ALL	Pre-T pre-B	10^8	0
CTCL-2	SS	Mature T	2×10^7	63
CTCL-2	SS	Mature T	5×10^7	98
HUT-78	MF	Mature T	5×10^7	91

* The designations are acute lymphoblastic leukemia (ALL), Burkitt's lymphoma (BL), histiocytic lymphoma (HL), Sézary syndrome (SS), and mycosis fungoides (MF).

‡ Mature T cells were E-rosette positive and TdT negative, whereas immature T cells were TdT positive. B cells were surface immunoglobulin positive. Nalm-1 had intracellular μ-chains and was TdT positive.

§ All adsorptions were carried out at 4°C for 2 h with the samples unable to adsorb being incubated for 2 additional h. The standard TCGF preparation stimulated [^{3}H]TdR incorporation of 18–25,000 cpm at maximum. The TCGF removed was determined as described in Materials and Methods.

TABLE III

Adsorptions Using Coincubation of TCGF and CSA

HUT-102 material	CSA activity*		TCGF activity‡	
	Colonies/ 2×10^5 cells	Percent remaining	Units/ml	Percent remaining
None	67	100	5.3	100
Cells§	63	94	0.2	3
Plasma membrane 1‖	64	96	0.5	10
Plasma membrane 2‖	55	82	0.8	16

* CSA activity was assessed using 2×10^5 nonadherent bone marrow cells per plate in methylcellulose (17). After 14 d of incubation, colonies of >50 cells were counted. The source of CSA was conditioned media prepared from human placenta cells under serum-free conditions[3] mixed with partially purified TCGF in a ratio of ~60 colonies/2×10^5 marrow cells to 5 U of TCGF (the standard preparation used in all the other adsorption experiments was given a value of 10 U/ml).

‡ TCGF activity was assayed as described in Materials and Methods. Incubations were performed at 4°C for 2 h in the presence of 0.1 mM PMSF, 1% trasylol, 1 mM TLCK, and 1 mM TPCK. Samples were extensively dialyzed against 1,000 times volume of PBS for 3 d before assay. The mixture of CSA and TCGF used was described above.

§ HUT-102 cells at a concentration of 2.5×10^7 cells/ml were used in an incubation with a mixture of CSA and TCGF as described above.

‖Cell membranes were prepared from HUT-102 cells preincubated with chloroquine as described in Materials and Methods. This membrane fraction contained 70% of the total 5′-thymidine-phosphodiesterase activity, a plasma membrane marker (20) (See Table VI). Plasma membrane fractions 1 and 2, containing 2 and 0.4 mg/ml protein, respectively, were incubated with a mixture of TCGF and CSA as described above.

TABLE IV

Screening of Human Lymphoma-Leukemia Cells for Constitutive Production of TCGF

Cutaneous T cell lymphoma*	TCGF activity‡	Cell lines with no activity§		
		T cell Leukemia	B cell lymphoma-leukemia	Non-T, non-B neoplasias
	[^{3}H]TdR cpm			
HUT-102	13,000 ± 3,800	CCRF-CEM	RAMOS	NALM-1
HUT-78	5,600 ± 1,700	CCRF-HSB2	RAJI	NALM-16
CTCL-2	3,600 ± 980	Molt-4	DAUDI	ML-1-3
Standard TCGF	18,000 ± 780	8402	RPMI 1788	U937
		T-45	RPMI 8866	KG-1
		PEER-1	LPN2	K562
		HPB-ALL	BJAB	HL-60
		HPB-MLT	EB-3	DHL-2
		JM		KM-3
		SKW3		REH
		TALL-1		NALL-1

* All cell lines were grown in RPMI 1640 with 10% FCS. The conditioned media were concentrated 10–20-fold by ammonium sulfate precipitation before assaying for TCGF.

‡ TCGF activity was measured using the microassay for [^{3}H]TdR incorporation as described in Materials and Methods. All media were first concentrated by ammonium sulfate precipitation. Experiments were done in triplicate ± SE.

§ These cell lines were all treated in an identical manner to CTCL cell lines. No detectable TCGF activity was found with and without stimulation with PHA at various concentrations.

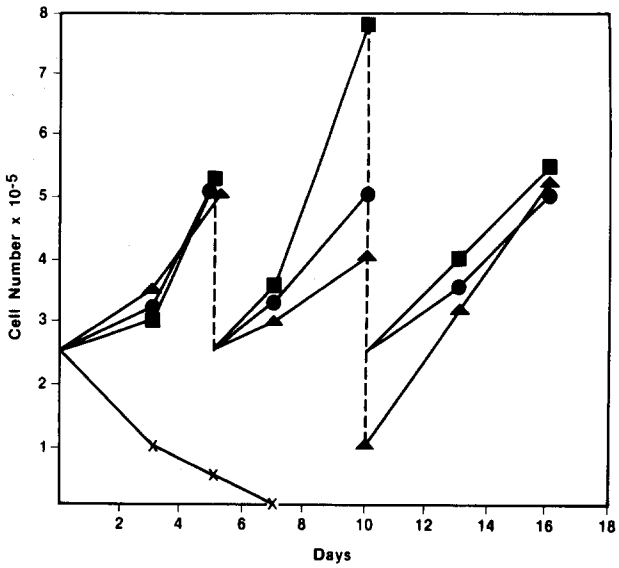

FIG. 4. Growth of cultured T cells using eluated or conditioned media from HUT-102. Cultured normal human T cells were first grown in N-TCGF for 22 d after an initial stimulation with PHA. The cells were washed with warm RPMI 1640 to remove residual TCGF and then used as target cells for TCGF assays as follows. They were cultured in RPMI 1640 containing 10% FCS and the following materials: HUT-102 acid eluate 50% (vol/vol) (■); a second HUT-102 acid eluate 50% (vol/vol) (●); HUT-102 conditioned media (▲); and control (no additive) (×). All HUT-102 material was concentrated 10-fold by ammonium sulfate precipitation before use. Cell counts were performed every 3–4 d.

Elution of TCGF from Cell Surfaces and Isolated Membranes of CTCL and PHA-stimulated Normal T Cells. Eluates were prepared from 1–2 g of various cell lines using an acid glycine buffer (pH 2.8). Only eluates from two CTCL lines (HUT-102 and HUT-78) and PHA-stimulated PBL stimulated incorporation of [^{3}H]TdR in the microassay for TCGF (Table V). CTCL-2 was not tested due to a difficulty in obtaining large quantities of cells. HUT-102 eluates were also to support the growth of T cells (Fig. 4), a more definitive test for TCGF than the [^{3}H]TdR incorporation microassay described above. Cells grown for over 3 wk in the eluate material were >90% E-rosette positive. The amount of TCGF eluted from the HUT-102 cells was cell concentration dependent and detectable at 2×10^8 cell/ml (Table V). TCGF activity was also found in the cell eluate (concentrated 100 times) made under conditions which maintained cell viability of >70% (10^7 cells/ml, 1 min incubation at 4°C in glycine buffer, pH 2.8).

Cell membranes prepared from HUT-102 cells were also eluted and the eluates yielded detectable TCGF activity (Table VI). The cell membrane fraction contained 70% of the phosphodiesterase I activity and 77% of the TCGF activity. The cytoplasmic fractions and nuclear membrane eluate contained minimal TCGF. However, the mitochondrial cell pellet, which probably contained some plasma membrane, also contained activity, but much less than the plasma membrane fraction having no succinate dehydrogenase activity (data not shown).

Growth of Activated but Not of Unstimulated T Cells by TCGF from Normal Activated T Cells and from CTCL Lines. A comparison of L-TCGF and N-TCGF showed that they possess similar biologic functions (Fig. 5). Freshly isolated PBL incubated in the presence of either L-TCGF eluates, CM, or N-TCGF CM for 3–7 d showed no increase in [^{3}H]TdR incorporation. The same PBL used after 5 d of PHA stimulation incorporated thymidine 75–100-fold above background in the presence of either L-TCGF or N-TCGF materials.

Enhanced Proliferation of TCGF-dependent and TCGF-independent CTCL Lines in Response

TABLE V

TCGF Activity Detectable by Acid Glycine Elution

Cells*	Cell number/ eluate‡	TCGF activity§
	no./ml	*[^{3}H]TdR cpm*
HUT-102	2×10^8	20,300 ± 2,600
HUT-78	2×10^8	5,100 ± 1,700
PHA-stimulated PBL	10^8	12,700 ± 2,100
HSB-2	5×10^8	150 ± 110
NALM-1	10^8	522 ± 152
Molt-4	2×10^8	270 ± 190
DAUDI	2×10^8	650 ± 190
Nonstimulated PBL	10^9	180 ± 30
Standard TCGF	—	9,800 ± 1,400

* All cell lines were grown on RPMI 1640 containing 10% FCS.

‡ An acid eluate was made using glycine buffer (pH 2.8) at 4°C for 10 min. After extensive dialysis, the elutates were assayed for TCGF.

§ TCGF activity was measured using the microassay for [^{3}H]TdR incorporation as described in Materials and Methods. All media were first concentrated by ammonium sulfate precipitation. Experiments were done in triplicate ± SE.

TABLE VI

Subcellular Localization of TCGF Activity in HUT-102 Cells

Cellular fraction	Phosphodiesterase I*			TCGF activity‡	
	Specific activity	Relative activity (lysate = 1.0)	Total recovered	[^{3}H]TdR uptake	TCGF recovered
	μM Nitrophenol/mg/h		%	*cpm*	%
Lysate	0.039	1.0	—	ND§	ND§
Cytoplasm	0.010	0.3	17.0	1,020	8.5
Nuclei pellet	0.016	0.4	7.5	35	1.3
Mitochondrial pellet	0.028	0.7	5.0	3,400	12.9
Plasma membrane	0.207	5.4	70.5	18,700	77.3

* Thymidine-5′-phosphodiesterase was measured using thymidine-5′-monophosphate-*p*-nitrophenol ester as substrate. Units expressed as micromoles *p*-nitrophenol released per hour at pH 9.0. Specific activity was calculated as units per milligram protein in sample. Relative specific activity was derived by dividing the specific activity of each sample by the specific activity of the lysate used as starting material for the fractionation. Total activity recovered was calculated by multiplying the enzyme units per 10 μl assay sample by the total volume of each sample and then the fraction of the total activity contained in each fraction was determined.

‡ TCGF activity of concentrates prepared by acid elution and $(NH_4)_2SO_4$ precipitation of nuclear, mitochondrial, and plasma membrane particulate fractions and $(NH_4)_2SO_4$ precipitation of soluble cytoplasmic fractions was tested in a [^{3}H]TdR incorporation microassay as described in Materials and Methods. Relative TCGF potencies were derived by comparing the slopes of the regression lines generated by serial $\log_2$ dilution of the samples TCGF recovered was calculated by multiplying the amount present in the assay by the total volume of the sample and expressed as a percentage of the total.

§ Cell lysate was used as starting material for fractionation and was not assayed for TCGF.

to Exogenous TCGF. Both factor-independent and factor-dependent CTCL were grown in the presence of added exogenous TCGF. The addition of TCGF to HUT-102 cells increased their incorporation of [^{3}H]TdR (Fig. 6) in a concentration-dependent manner. The rate of proliferation of CTCL-2, another TCGF-independent CTCL cell line, increased during incubation with either L-TCGF or N-TCGF (Table VII). TCGF-dependent CTCL and acute lymphocytic leukemia cells proliferated in response to TCGF- and L-TCGF-containing acid eluates from HUT-102 in an equally efficient manner (Table VII).

Discussion

The addition of TCGF to T cells previously activated by antigen or lectin has allowed the routine long-term growth of mature normal human T cells (1, 2). Lymphocyte activation by antigen or lectin involves two distinct steps: stimulation of TCGF release and development of TCGF-responsive T cells. The development of the TCGF-responsive state appears to be mediated by the appearance of TCGF-specific membrane binding sites. Consistent with this model, no detectable TCGF activity remains in the conditioned media of cultured T cells 72–96 h after the addition of TCGF (8), suggesting that the proliferating T cells inactivate or bind and remove TCGF from the media. It has been previously shown that T cells activated by lectin or antigen remove TCGF activity in a time-, temperature-, and cell concentration-dependent manner whereas unstimulated T cells or cells stimulated with a B cell mitogen do not (6–8). Under the conditions described here, freshly isolated mononuclear cells removed some TCGF activity but only at high cell concentrations (5×10^8

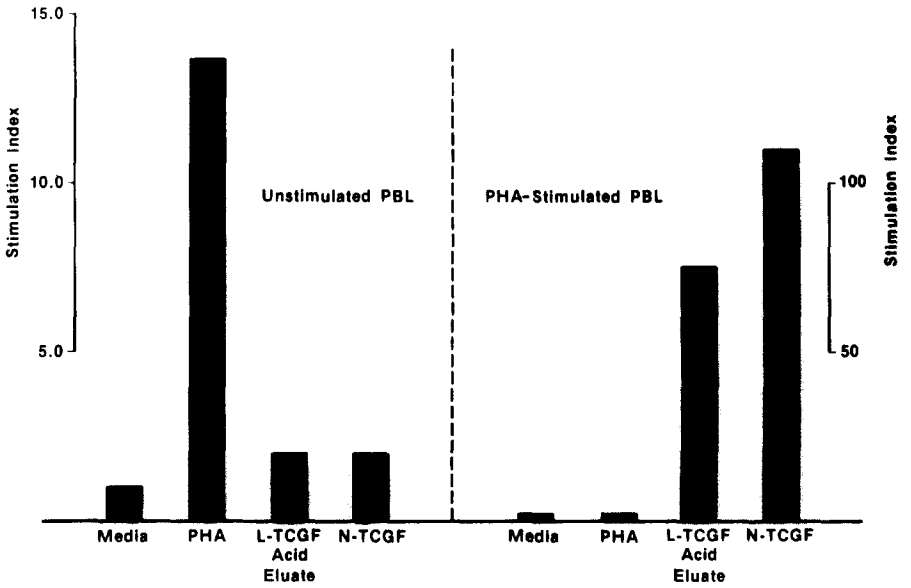

FIG. 5. Comparison of biologic activities of N-TCGF and L-TCGF. (Panel A) Unstimulated normal human PBL were incubated in media supplemented with PHA-P (10 μl/ml); N-TCGF (10% vol/vol), or L-TCGF (20% vol/vol) for 3 d before [^{3}H]TdR incorporation was determined. (Panel B) PBL were stimulated with PHA for 5 d. After washing, the cells were incubated in media supplemented with PHA-P (10 μl/ml), N-TCGF (10% vol/vol), or L-TCGF (20% vol/vol) for 3 d before [^{3}H]TdR incorporation was determined.

cells removed 18% of the TCGF activity). This may be due to the presence in peripheral blood of a minor population of activated T cells. In contrast, T cells activated either in vitro by PHA or alloantigen or in vivo in the case of patient with IM completely adsorbed TCGF activity using only 5×10^7 cells/ml.

In contrast to TCGF adsorption mediated by normal antigen or lectin-activated T cells and similar to the resslts with T cells derived from a patient with IM, cells lines derived from patients with cutaneous T cell lymphoma and leukemia adsorbed TCGF without any apparent in vitro activation signal. For instance, HUT-102 cells removed all TCGF activity at a cell concentration of 2×10^7 cells, whereas 5×10^7 normally activated T cells per ml were required to remove all activity using identical TCGF preparations. It is not known whether this is a result of differences in the affinity of TCGF receptor binding, or receptor density per cell, or both. Incubation at 4°C which markedly inhibits endocytosis and many other metabolic functions, and the addition of metabolic, proteolytic, and lysosomal function inhibitors did not affect the removal of TCGF activity. Removal of activity seems specific for cell lines of mature T cell origin because normal B cells, neoplastic B cells, neoplastic immature T cells, neoplastic pre-B cells, myeloid, and monocytoid cells failed to remove TCGF activity. The evidence that the removal of TCGF was not due to a nonspecific event such as TCGF degradation was not limited to the cell specificity. When normal T cells and the CTL cell lines were incubated with a mixture of TCGF and CSA, a growth factor specific for myeloid cells, all of the TCGF activity and none of the CSA activity was removed. In addition, incubation of HUT-102 plasma membrane fractions with TCGF and CSA in the presence of proteolytic inhibitors gave results

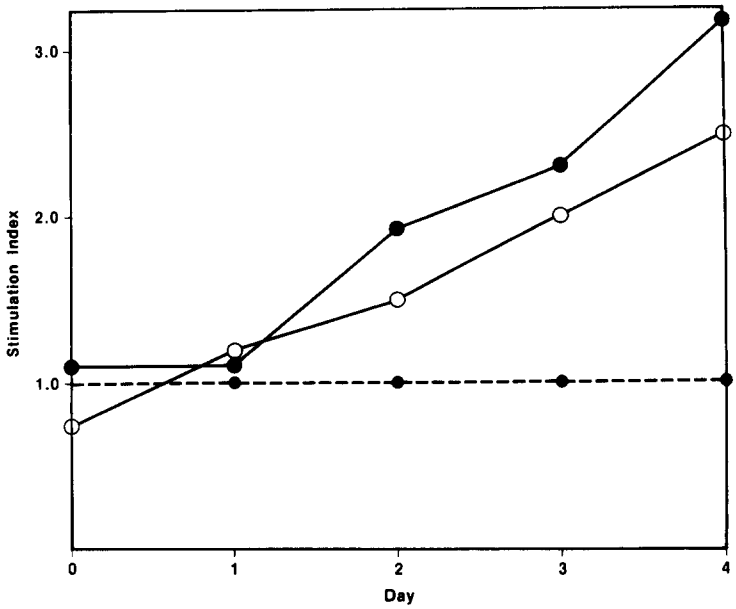

FIG. 6. Response of HUT-102 to added TCGF. HUT-102 were cultured at an initial concentration of 5×10^4 in RPMI 1640 containing 10% FCS with or without N-TCGF at either 20% (○) or 60% (●) vol/vol. The media were replenished daily. [^{3}H]TdR incorporation was determined on aliquots daily. A stimulation index was determined as described in Materials and Methods. The flat line (also shown as solid circles, ●) represents HUT-102 cells grown in the absence of added TCGF and represents normalized (100%) growth.

TABLE VII

Proliferation of Malignant T Cells in Response to N-TCGF and L-TCGF

Cells*	TCGF dependence	Growth saturation density‡		
		Media	N-TCGF	L-TCGF
CTCL-14	Yes	Dead	1.2×10^6	1.2×10^6
ALL-16	Yes	Dead	1.0×10^6	1.0×10^6
CTCL-2	No	2×10^5	5.0×10^5	4.0×10^5

* CTCL-2 was derived from a patient with Sézary syndrome. It was initiated with TCGF but was grown independent of TCGF for 26 wk before use. CTCL-14, derived from a patient with mycosis fungoides, and ALL-66, derived from a patient with T cell acute lymphoid leukemias, were initiated and maintained in culture using partially purified TCGF for 5 and 10 wk, respectively, before use.

‡ All cell lines were put in culture at 10^5 cells/ml in RPMI 1640 media containing 10% FCS. Either TCGF was added at 50% (vol/vol). Cell counts were performed daily until the saturation density of the culture was reached in 5–7 d. L-TCGF was derived from acid elution of HUT-102 cells, and N-TCGF was derived from conditioned media from PHA-stimulated normal lymphocytes.

identical to those obtained with whole cells. These results strongly suggest that there are specific TCGF binding sites on the cell surface of both normal activated T cells and human neoplastic T cell lines of mature T cell origin.

Finally, the presence of specific binding sites for TCGF on the CTCL lines is also

suggested by the presence of TCGF activity in the acid eluates from both HUT-102 and HUT-78 cells. This TCGF appears to be associated with the cell surface since TCGF activity was also eluted from partially purified plasma membrane fractions and from cells that retained their viability after the elution procedure. The eluted activity supported the growth of cultured T cells, whereas eluates from several other cell lines had no activity. These results strongly suggest that there are specific TCGF binding sites on the cell surface of both normal activated T cells and human neoplastic T cell lines of mature T cell origin.

The TCGF found on the cell surfaces of these CTCL lines is also present in the conditioned media of HUT-102, HUT-78, and CTCL-2. These three CTCL cell lines are the first human cell lines known to constitutively release TCGF. A malignant T cell line developed from a Gibbon ape spontaneously releases TCGF.[4] A few murine tumor cell lines and one human cell line have been reported to produce TCGF but only upon induction with lectins and phorbol esters (23–25). A survey of other human tumor cell lines including several immature leukemic T cell lines did not reveal any additional cell lines that could release TCGF either constitutively or upon induction. These results are consistent with both the helper T cell nature of CTCL (26) and the evidence that the normal TCGF producer cell is a helper T cell (27, 28).

The production of TCGF, the apparent presence of membrane receptors for TCGF, and the recovery of TCGF from the cell surface of these CTCL lines all suggest that these cell lines proliferate in response to endogenously produced TCGF. Addition of N-TCGF to HUT-102 cells augmented [^{3}H]TdR incorporation in a dose-dependent manner. Addition of either L-TCGF or N-TCGF to CTCL-2 (another TCGF-independent cell line) and to various TCGF-dependent T cell lines of neoplastic origin resulted in increased proliferation of these cells. The results taken together indicate that these CTCL lines both produce and respond to L-TCGF. No normal T cell line has been shown both to constitutively produce and respond to TCGF (3, 4, 27). Cloned murine helper T cells, which respond to TCGF, produced TCGF only when histocompatible macrophages and specific antigen are added (27).

The proliferation of CTCL may be due to the continuous production of TCGF by the same cell that expresses TCGF receptors on its cell surface. This is analogous to studies of growth regulations of anchorage-dependent tumor cell lines in which a few human tumor cell lines constitutively release protein factor(s) that stimulate growth of normal and malignant human cell lines by binding to the normal epidermal growth factor receptor (29). In these systems, it has been suggested that malignant cells "autostimulate" their growth by producing and responding to these protein growth factor(s) (30). Single-cell cloning experiments using these cell lines are in progress to unambiguously verify whether the same cell produces and responds to TCGF. However, even if this is found it does not necessarily indicate that "self-stimulation" is an essential aspect of neoplasia. In fact, it is much more frequent to find neoplastic mature T cells that maintain a requirement for exogenous TCGF for in vitro growth. Therefore, constitutive TCGF production and response by the same cell could simply be one of many unusual phenotypic variant properties of neoplastic cells which, when it occurs, gives them a selective advantage for in vitro growth.

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[4] Rabin, H., R. F. Hopkins III, F. W. Ruscetti, R. H. Neubasuer, R. L. Brown, and T. G. Kawakami. A T cell tumor line derived from a Gibbon ape spontaneously releases T cell growth factor activity. Submitted for publication.

Summary

Three cell lines of mature T cell origin derived from patients with cutaneous T cell lymphoma-leukemias (CTCL) were found to be constitutive producers of T cell growth factor (L-TCGF). These are the first reported human cell lines which constitutively produce TCGF. Biologically active TCGF could also be eluted from the surface of these cells using an acid glycine buffer under conditions that maintained cell viability, and subcellular fractionation showed that almost all the TCGF activity was associated with the plasma membrane. Over 30 other human hematopoietic cell lines derived from other disorders were unable to produce TCGF even after induction, and their acid eluates did not contain TCGF activity. L-TCGF from CTCL lines had the same biological activity as TCGF obtained from normal leukocytes (N-TCGF) in that they both supported the long-term growth of normal T cells only after the cells were previously activated by antigen or lectin. Both L-TCGF and N-TCGF increased the rate of proliferation of TCGF-independent and TCGF-dependent CTCL cell lines. The same three factor-independent cell lines that released TCGF adsorbed TCGF in a cell-concentration, time-, and temperature-dependent manner. Since the CTCL cell lines produce TCGF, adsorb TCGF, and increase their proliferative rate in response to TCGF or a related molecule, it is suggested that this endogenously produced factor plays a role in maintaining the abnormal proliferation of these cells in culture as permanently growing cell lines independent of exogenous TCGF. However, this does not mean that this is an essential aspect of neoplastic transformation. Since it is unusual to develop these cell lines in the absence of the continuous need for added TCGF, "autostimulation" may be one of the many unusual variant phenotypic properties sometimes associated with neoplastic cells that gives them a selective advantage for in vitro growth.

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