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T-cell lines established from human T-lymphocytic neoplasias by direct response to T-cell growth factor

(mycosis fungoides/Sezary syndrome/acute lymphocytic leukemia)

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ABSTRACT **Long-term growth of lymphoblastoid T cells from tissue samples from six of six patients with cutaneous T-cell lymphoma (CTCL) and six of six patients with acute T-lymphoblastic leukemia (ALL) has been achieved by using partially purified mitogen-free human T-cell growth factor (pp-TCGF). One cell line, CTCL-2, is now independent of added growth factor; the others continue to show absolute dependency on its presence. All lines have been in continuous culture for at least 4 months and some for >1 year. They are erythrocyte-rosette positive and are negative for Epstein–Barr virus nuclear antigen. Most of the lines are negative for Fc and complement receptors and for surface immunoglobulin except that CTCL-1 and CTCL-2 have some cells positive for these cell surface markers. Results of histochemical studies on these cell lines are similar to the known patterns for fresh cells from their disease of origin. Cell line CTCL-3 has an abnormal karyotype, but no detectable chromosomal abnormalities were found in the other lines, consistent with the karyologic features of their clinical sources. Because T cells from normal donors do not respond to pp-TCGF unless the cells are first "activated" by a lectin mitogen such as phytohemagglutinin or an antigen, the direct response to pp-TCGF of T cells from patients with T-cell neoplasias suggests that the cell lines represent a transformed neoplastic cell population. Although some of the cell lines may be normal T cells activated by the malignant cells, the morphologic and histochemical properties of the cell lines, the abnormal karyotype of CTCL-3, and the independent growth of CTCL-2 support the conclusion that most of these cell lines are of malignant origin.**

Medium conditioned by mitogen-stimulated human mononuclear cell cultures contains a T-cell growth factor (TCGF) (1, 2). Such crude media will support the growth of normal human T lymphocytes from either peripheral blood or bone marrow in liquid suspension for prolonged periods, but no TCGF-independent normal T-cell line has been established despite several samples being in culture for more than 13 months (2). By using crude TCGF or mitogen stimulation, two cell lines from samples of patients with cutaneous T-cell lymphoma (CTCL) were established (3), but this was indeed a rare event because most attempts resulted in either short-term growth or the establishment of an Epstein–Barr virus nuclear antigen (EBNA)-positive B-lymphoblast cell line. A few lymphoblastoid lines (e.g., Molt 4, CCRF-CEM, 8402) have been established from cases of acute lymphocytic leukemia (ALL) that apparently are independent from TCGF. These cell lines have all been terminal deoxynucleotidyltransferase-positive (a marker for immature lymphoblasts but not restricted to the T-lymphoid lineage) and are, in general, erythrocyte-rosette-receptor negative.

Substantial purification of human TCGF has been achieved recently (4). The active fraction is free of interferon, colony-stimulating activity, and mitogenic activity for fresh lymphocytes obtained from normal donors but will support the growth of these lymphocytes after activation by a mitogen or antigen (4). This paper reports the use of a partially purified fraction of the TCGF in the long-term growth of T cells from various human lymphocytic neoplasias.

MATERIALS AND METHODS

Preparation of TCGF. The detailed purification scheme and properties of the purified TCGF are described elsewhere (4). Here we summarize the basic steps for making the partially purified human TCGF (pp-TCGF) used in this study.

Crude TCGF was prepared by incubating human lymphocytes from multiple healthy donors at a density of 10^6 cells per ml in RPMI-1640 medium in the absence of serum but containing 0.25% bovine serum albumin and 10 μg of phytohemagglutin (PHA-P; Difco) per ml at 37°C for 72 hr. (1). The cells were removed by centrifugation and the medium was concentrated by $(NH_4)_2SO_4$ precipitation. Sufficient $(NH_4)_2SO_4$ to yield a 50% saturated solution was added to the crude medium; after stirring for 1 hr at 4°C, the suspension was centrifuged at 10,000 × g for 10 min. Additional $(NH_4)_2SO_4$ was added to the supernatant to produce a 75% saturated solution, the mixture was stirred for 1 hr at 4°C, and the suspension was centrifuged as above. The precipitate was dissolved in a minimal amount of 0.01 M Tris·HCl (pH 8.0) (buffer A) at 4°C and dialyzed against 50 vol of the same buffer for 14 hr at 4°C. Subsequently, the concentrate was placed on a DEAE-Sepharose column equilibrated with buffer A, and the adsorbed proteins were eluted with a linear 0–0.2 M NaCl gradient in buffer A. The TCGF activity eluted at approximately 0.07 M NaCl. The active fractions were pooled and diluted 1:1 with RPMI-1640/0.1% polyethylene glycol. This product, pp-TCGF, lacks colony-stimulating activity, interferon, detectable PHA activity, and direct mitogenic activity for fresh peripheral blood lymphocytes. Further purification and characterization of human TCGF are described elsewhere (4).

Establishment of Cell Lines. Heparinized peripheral blood or bone marrow samples from 12 patients (Table 1) were centrifuged at 1000 × g for 20 min, and the cell pellets were resuspended in RPMI-1640 medium. The mononuclear cells were isolated by using lymphocyte separation medium (Litton Bionetics, Kensington, MD) and, after being washed twice with medium, they were resuspended in RPMI-1640 containing L-glutamine, 20% fetal calf serum (Reheis, Phoenix, AZ), and 1% penicillin/streptomycin mixture. The cells were then placed in duplicate 25-cm^2 tissue culture flasks in a final volume of 5 ml containing 10^6 cells per ml under the following conditions:

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Abbreviations: TCGF, T-cell growth factor; pp-TCGF, partially purified T-cell growth factor; PHA, phytohemagglutinin; CTCL, cutaneous T-cell lymphoma; ALL, acute lymphocytic leukemia; EBNA, Epstein–Barr virus nuclear antigen.

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Table 1. Patients with T-lymphocytic neoplasias

Sample designation	Diagnosis	Source*	Age, yr; sex	Peripheral leukocytes, no. $\times 10^{-3}/mm^3$	Clinical status
CTCL-1	Sezary syndrome	PB	60; F	100	Untreated
CTCL-2	Sezary syndrome	PB	60; F	300	Untreated
CTCL-3	Mycosis fungoides	PB	28; M	7	Remission†
CTCL-4	Mycosis fungoides	BM	69; M	8	Untreated
CTCL-5	Mycosis fungoides	PB	69; M	10	Remission
CTCL-6	Mycosis fungoides	PB	66; F	8	Relapse†
ALL-1	ALL	BM	47; M	6	Untreated
ALL-2	ALL	BM	19; M	6	Untreated
ALL-3	ALL	PB	16; F	91	Untreated
ALL-4	ALL	PB	65; M	127	Untreated
ALL-5	ALL	PB	69; M	214	Untreated
ALL-6	ALL	PB	30; M	36	Untreated

*PB, peripheral blood; BM, bone marrow. The samples designated ALL-3, -4, and -5 were obtained by leukophoresis.
†After systemic chemotherapy and electron-beam radiation therapy.

(*i*) RPMI-1640 with 20% serum; (*ii*) RPMI-1640 with 20% serum and 50% crude TCGF; and (*iii*) RPMI-1640 with 20% serum and 20% pp-TCGF. The flasks were observed twice weekly, and cell number and viability were monitored periodically. If a sample achieved a concentration of 2×10^6/ml, it was diluted with an equal amount of its medium and continued in culture. If continuous culture was achieved with added growth factor, routine attempts were made to grow the cells in RPMI/serum without TCGF. PHA-stimulated normal peripheral blood lymphocytes (NPB) were also grown as cell lines with pp-TCGF, as above, and used as controls.

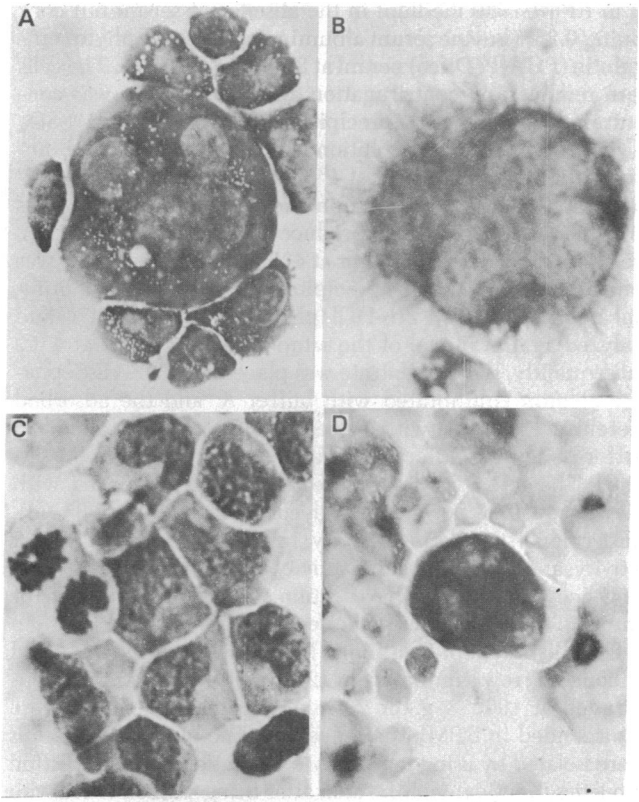

FIG. 1. Light microscopic appearance of cultured CTCL and ALL cells. (*A*) Cytocentrifuge preparation of cultured CTCL-2 cells, illustrating the varying size and nuclear number of cultured CTCL cells. (Wright–Giemsa stain; ×200.) (*B*) Example of the subpopulation of large, multinucleated cells with marked cytoplasmic projections found in cultured CTCL cells. (Wright–Giemsa stain; ×200.) (*C*) Cultured ALL-2 cells, illustrating the more uniform lymphoblast morphology seen with the ALL lines. (Wright–Giemsa stain; ×450.) (*D*) Positive nonspecific esterase stain on cultured CTCL-2 cells, showing the intense diffuse cytoplasmic reaction found in the large multinucleated cells and the more common pattern of a large paranuclear granule with or without diffuse staining found in smaller cells. (×200.)

Histochemical Staining Procedures. Naphthol ASD chloroacetate and α-naphthol acetate esterases (nonspecific esterase) (5), Sudan black B and alkaline phosphatase (6), myeloperoxidase (7), acid phosphatases with or without tartrate treatment (8), and the periodic acid/Schiff reaction (9) were assayed according to published procedures.

Cell Surface Studies and Test for Epstein–Barr Virus. Erythrocyte-rosette tests were performed by slight modifications of established techinques, as detailed elsewhere (10–12). 2-*S*-aminoethylisothiouranium bromide-treated sheep erythrocytes were used in the assays. Fc and complement receptors were estimated by using the antigen-coated sheep erythrocyte- and antigen- and complement-coated sheep erythrocyte-rosette tests performed with sheep erythrocytes. Tests for surface IgG, IgM, and IgA (SIg) were performed by using an immunofluorescent method, as detailed elsewhere (13). The EBNA test was performed by a modification of the Reedman–Klein technique (14).

Cytogenetic and Electron Microscopic Studies. Karyotype analyses were carried out initially on all fresh samples and periodically thereafter on the established cell lines according to published methods (15). Cell samples were also periodically removed from the cultures and centrifuged, and the cell pellets were prepared for thin-section electron microscopy.

RESULTS

Growth Characteristics of Established Cell Lines. T-cell lines were established from all 12 samples, but only when they were grown with pp-TCGF. When cell cultures were supplemented with RPMI/fetal calf serum or even with crude TCGF, the cells proliferated for only 1–3 weeks or 3–6 weeks, respectively (except for cell line ALL-4A, which proved to be a spontaneously transformed EBNA-positive B-cell line grown in RPMI/fetal calf serum). In contrast, fresh cells from T-cell neoplasias began to proliferate within 24–48 hr after stimulation with pp-TCGF, and these cell lines were maintained in culture with pp-TCGF for 4 months in all cases and for nearly a year in two cases. All remained dependent upon pp-TCGF except for CTCL-2 which was successfully transferred onto RPMI/fetal calf serum alone at its 10th passage (week 5). The doubling times of the cell lines varied from 24 to 72 hr. The CTCL cell

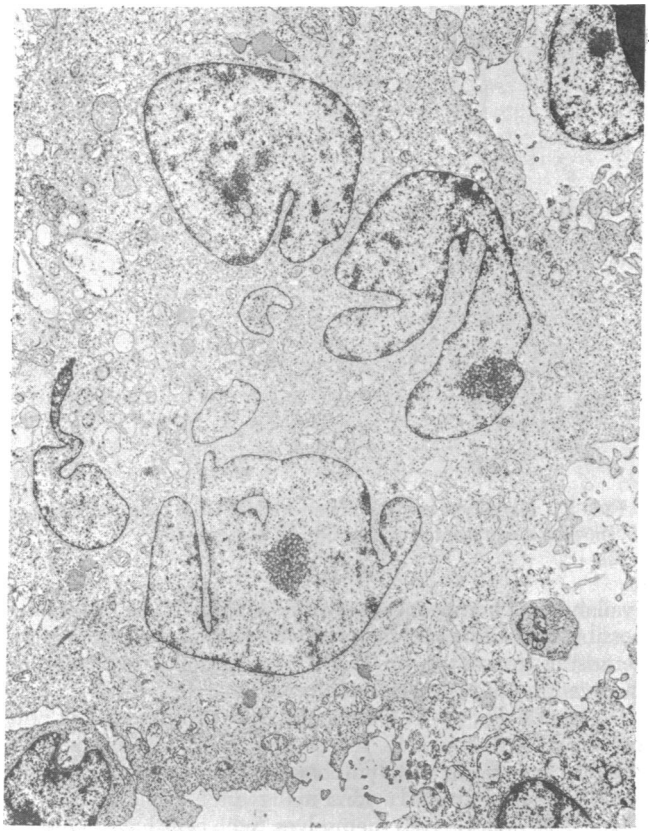

FIG. 2. Electron micrograph of cultured CTCL-3 cells showing a multinucleated cell with moderately convoluted nuclei. Cells were fixed for 1 hr at 20°C in 25% glutaraldehyde/0.1 M Na cacodylate, pH 7.3, washed five times at 4°C in 0.2 M Na cacodylate buffer (pH 7.3), and postfixed for 45 min at 4°C in Dalton's chrome osmium. After dehydration in graded ethanols, the cell pellets were embedded in Spurr low-viscosity embedding medium. Sections were cut on an LKB ultramicrotome and examined with a Siemens Elmiskop 102 at 80 LV. The small lymphocyte seen in the lower right is approximately 8 μm in diameter.

lines grew as large tight clumps of as many as thousands of cells; the ALL cell lines grew predominantly as single-cell suspensions. Normal PHA-stimulated T-cells grown with pp-TCGF grow as small tight clumps.

Morphologic and Cytochemical Characteristics of Cultured Cells. The CTCL cell lines differed from the ALL lines in some structural and cytochemical features. The cell size and number of nuclei per cell in the CTCL lines were variable, with many smaller mononucleated or binucleated cells clustering around multinucleated "giant cells" (Fig. 1*A*). The incidence of these large multinucleated cells ranged from 1% to 15% in the CTCL lines; CTCL-2 had the most. These cells often had marked cytoplasmic projections (Fig. 1*B*), giving them a "hairy" appearance. Wright–Giemsa stains and electron micrographs of CTCL cells showed them to have pale nuclei with finely dispersed chromatin and usually one rather indistinct nucleolus (Figs. 1*A* and 2). None of the CTCL lines demonstrated the feature of markedly convoluted nuclei characteristic of the peripheral lymphocytes of patients with Sezary syndrome, but they did contain some cells with moderately convoluted nuclei (Fig. 2), as is often seen in T cells from patients with mycosis fungoides (16). The ALL cell lines were more uniform in appearance, with most cells having a single nucleus with coarse chromatin and one or two prominent nucleoli (Fig. 1*C*).

The histochemical staining pattern of these cultured T cells remained constant throughout the culture period (Table 2). All the cultured T lymphoblasts, whether from normal donors or patients with T-cell neoplasias, were negative for Sudan black, myeloperoxidase, chloroacetate esterase, and alkaline phosphatase. A minority of cells from each sample type were positive with periodic acid/Schiff and this stain was of little use in discriminating among the three types of cell lines. All three cell types were positive for acid phosphatase, which was sensitive to tartrate treatment, but the staining pattern for each was distinct. The majority of cells in the ALL lines showed accentuation of the staining reaction in the Golgi region of the cell; the CTCL cells showed a diffuse cytoplasmic granular staining, more prominent in the large multinucleated giant cells. The cultured cells from normal donors exhibited a mild granular cytoplasmic reaction. Only the CTCL lines stained for nonspecific esterase in the procedure used. The reaction was diffusely intense in some cells from these samples (invariably, the large multinucleated cells with prominent cytoplasmic projections); most cells had a single large paranuclear cytoplasmic granule with or without some diffuse cytoplasmic staining (Fig. 1*D*).

Table 2. Histochemical-staining patterns of T lymphoblasts cultured from T-cell neoplasias and normal peripheral blood

Stain	Mean % positive cells* CTCL	ALL	NPB
Nonspecific esterase	100†	0	0
Acid phosphatase	100‡	100§	60¶
Tartrate/acid phosphatase	0	0	0
Periodic acid/Schiff	15	10	15
Sudan black B	0	0	0
Myeloperoxidase	0	0	0
Chloroacetate esterase	0	0	0
Alkaline phosphatase	0	0	0

* All CTCL and ALL cell lines and four T-cell lines from normal peripheral blood (NPB) were tested for their histochemical staining patterns after 21 days in culture and every 2 weeks thereafter for at least 4 months in culture. The staining patterns remained constant throughout this culture period.
† A diffusely intense reaction in a minority of the cells; the majority had a single large paranuclear cytoplasmic granule with or without some diffuse cytoplasmic staining.
‡ Heavy diffuse granular pattern.
§ Reaction concentrated in the Golgi region.
¶ Light diffuse granular pattern.

Cell Surface Markers and EBNA. Except for ALL-4A, all the cell lines were erythrocyte-rosette positive and were negative for EBNA. Most lines were negative for Fc and complement receptors and for surface immunoglobulin. These characteristics of T cells were retained during long-term growth (Table 3). Cell lines CTCL-1 and CTCL-2 were consistently positive for Fc receptors, complement receptors, and surface immunoglobulins. Some of the CTCL lines varied in the degree of erythrocyte-rosette formation from one passage to another. This phenomenon may be a function of cell cycle and occurred whether or not a cell line required TCGF for growth (Table 4).

Cytogenetic Analysis. Cytogenetic studies were done on all samples before and after growth in culture. The cultured CTCL lines had an incidence of polyploidy that ranged from 1% to 15%, which was consistent with the percentage of multinucleated cells observed on light microscopy. All specimens except CTCL-3, however, had normal metaphases. Fresh and cultured CTCL-3 metaphases were hypodiploid, pseudodiploid, and diploid with a wide variety of karytotypic abnormalities, the most frequent of which was absence of chromosome 16 (Fig.

Table 3. Cell surface markers of EBNA of cells cultured from T-cell neoplasias and normal peripheral blood

Cell line*	% of cells positive†				
	E	EA	EAC	SIg	EBNA
CTCL-1	83–87	18–37	52–63	5–10	0
CTCL-2	26–80	22–28	10–16	8	0
CTCL-3	43–93	0	0	0	0
CTCL-4	83–93	0	0	0	0
CTCL-5	78–86	0	0	0	0
CTCL-6	72–85	0	0	0	0
ALL-1	59–72	0	0	0	0
ALL-2	52–97	0	0	0	0
ALL-3	79–93	0	0	0	0
ALL-4	29–41	0	0	0	0
ALL-4A	8–10	0	50	48	90
ALL-5	85–91	0	0	0	0
ALL-6	50–70	0	0	0	0
NPB	80–100	5	0	0	0

* All the cell lines were grown with pp-TCGF except CTCL-2 (which had become independent of added pp-TCGF) and ALL-4A (which grew spontaneously in RPMI/fetal calf serum alone). The cell lines were grown in pp-TCGF for 15–21 days before initial testing for surface markers. CTCL-1, CTCL-4, ALL-1, and ALL-4 were tested on days 15 and 30; all other cell lines were tested every 2 weeks during the first 4 months in culture.

† Erythrocyte (E)-, antigen-coated erythrocyte (EA)-, and antigen- and complement-coated erythrocyte (EAC)-rosettes, surface immunoglobulin, and EBNA test results are expressed as percentage of cells scoring positive. The erythrocyte-rosette test varied from one passage to another, and results are expressed as ranges.

3). This result is compatible with the lack of cytogenetic clone formation characteristic of the CTCLs (17). The data taken as a whole show a close correlation between the karyotypes of the fresh and cultured cells.

DISCUSSION

Implications of Growth with pp-TCGF. The data reported here prove that pp-TCGF can support the long-term growth of T cells from peripheral blood or bone marrow from patients with various T-lymphocytic neoplasias without prior stimulation with mitogen or antigen. This is in striking contrast to our results with T cells derived from normal donors. In the latter, the T cells must be activated by mitogen or antigen before they can be grown with pp-TCGF (4). The fact that crude TCGF is less efficient than pp-TCGF for continued proliferation suggests that the continued stimulation with mitogen present in the crude material may be toxic to transformed T cells. Alternatively, other factors (e.g., interferon) present in unpurified lymphocyte-conditioned medium may select against a malignant population. PHA, colony-stimulating activity, and interferon were not able to maintain the growth of these cell lines. However, because we used pp-TCGF due to the limited availability of homogeneous purified human TCGF, it remains possible that additional factor(s) with similar molecular characteristics (present in pp-TCGF preparations) might also be involved in producing the results we have described.

Table 4. Variability of erythrocyte-rosette formation

Cell line	TCGF	% positive			
		Confluent	Day 1	Day 2	Day 3
CTCL-2	−	26	45	59	66
CTCL-5	+	81	95	92	85
NPB	+	75	95	86	80

Cells were grown with or without TCGF to a confluent state (2×10^6 cells per ml). The percentage of cells forming erythrocyte-rosettes was determined, and the cells were resuspended in fresh medium at 1×10^5 cells per ml. The erythrocyte-rosette test was then performed on the first, second, and third days thereafter. Results are expressed as the percentage of lymphoblasts with greater than five sheep erythrocytes bound to their surface.

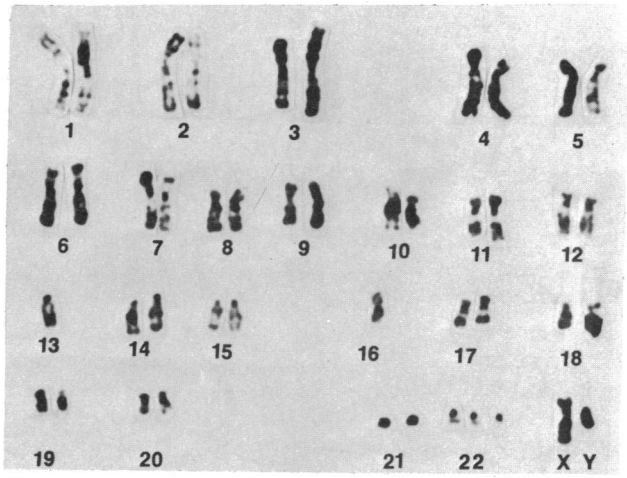

FIG. 3. Karyotype analysis of cultured CTCL-3 cells after passage 15, showing a representative hypodiploid pattern (45; X, Y, −13, −16, +22).

Theoretically, pp-TCGF should cause proliferation of only previously activated cells. It is possible, then, that the cells derived from patients with lymphoid malignancies are cytotoxic normal T cells activated against malignant lymphoblasts rather than the neoplastic cells themselves. Such cells have been cultured by using crude TCGF (18, 19), and recent experiments in our own laboratory show that cytotoxic T cells can be cultured from the peripheral blood of patients with acute myelogenous leukemia by using pp-TCGF (20). Although our results are not sufficient to prove unambiguously that the cells established in culture are normal activated T cells or neoplastic T cells, that the cell lines represent neoplastic cell populations is supported by the facts that: (*i*) CTCL-2 grows independently of TCGF; (*ii*) CTCL-3 has an abnormal karyotype; and (*iii*) the histochemical patterns of each of the cell lines are unique and consistent with published results on fresh cells from patients with CTCL or T-cell ALL (21–23). The unique, large, multinucleated cells with pronounced cytoplasmic projections observed in the CTCL lines are interesting. Their diffuse intense staining patterns for nonspecific esterase and acid phosphatase suggest that they may be monocyte-macrophage derived (5), but many of these cells do form erythocyte-rosettes with sheep erythrocytes (data not shown) and hence have T-cell derivation. The sensitivity of the acid phosphatase reaction to tartrate treatment confirms that these unusual cells do not represent the cells seen in hairy cell leukemia (23). Conceivably, they represent neoplastic T cells with aberrant properties: further investigation is required.

We have proposed that TCGF is a second signal for sustained growth of previously activated normal T lymphocytes (24). Presumably, antigen or mitogen stimulation induces cell membrane alterations to produce or expose a receptor(s) for TCGF. Neoplastic T cells may have such a receptor(s) on their cell surface at all times, thereby explaining the ability to grow T cells from CTCL and ALL samples with pp-TCGF without prior mitogen stimulation. This could be due either to chronic stimulation by some ill-defined antigen or to cell membrane changes which lead to exposure of a receptor or its synthesis *de novo*. If TCGF plays a role in the *in vivo* regulation of T-cell replication, as seems most probable, the above model may explain a growth advantage of malignant lymphocytes over normal T cells.

Some Uses of pp-TCGF and the Established Cell Lines. *Prediction of the presence of neoplastic T cells.* The karyotypically abnormal line CTCL-3 was established when the donor was thought to be in clinical remission from mycosis fungoides. At that time, no convoluted cells could be observed in his peripheral blood by either light or electron microscopy. A month later, this patient relapsed and his peripheral blood contained neoplastic T cells. It is intriguing to speculate that his impending recurrence was predicted by the growth of CTCL-3 with pp-TCGF. Routine attempts to grow malignant cells with pp-TCGF at times of clinical remission might prove prognostic, both in this disease and in T-cell ALL. Because in ALL, where the achievement of true complete remission is so important to ultimate survival (25), growth of such cells might indicate the use of further intensive chemotherapy, whereas routine cytochemical examinations of marrow specimens might erroneously suggest a switch to the form of therapy used when these patients are in remission.

Cell systems for comparative studies of normal and neoplastic T cells. The CTCL and ALL cell lines provide easily grown cells for comparison with similarly cultured, mitogen-stimulated normal T lymphoblasts. Disease-specific cellular enzyme levels or cell surface markers might be defined by comparisons between the two. A case in point would be the differences seen with the acid phosphatase and nonspecific esterase stains. Our results with cultured cells greatly exaggerate the differences between fresh peripheral normal T lymphocytes and Sezary cells that have been reported (22, 23). If such results are consistent, the nonspecific esterase stain could become an important aid in the diagnosis of the CTCLs. Another important area for study would be a comparison of the functional capabilities of the normal and neoplastic cells. The critical interactions, both cellular and humoral, important in the regulation of their own growth and that of other cell types can be more clearly defined.

In addition to providing an opportunity to define cellular differences between normal and neoplastic T cells, these neoplastic cells offer a unique opportunity to look for etiologic agents. Because RNA-directed DNA polymerase has been found in some ALL cells (26), and retroviruses are often fully formed only in proliferating cells (27), the cell lines described in this report should be examined for the *in vitro* expression of retroviruses.

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