Brief Definitive Report

LONG-TERM CULTURE OF HUMAN ANTIGEN-SPECIFIC CYTOTOXIC T-CELL LINES*

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One of us (F.R.) recently reported a method allowing for the long-term culture of normal human T lymphocytes from peripheral blood or bone marrow (1). Exponential proliferative growth was dependent upon the addition of a T-cell growth factor (TCGF) produced by phytohemagglutinin-stimulated normal human lymphocytes (1, 2). We have used similar methodology and have reported the long-term culture of murine tumor-antigen specific cytotoxic T-cell lines (CTLL) (3). Murine CTLL have remained in culture for over 20 mo during which time they have continued to mediate syngeneic tumor-specific cytotoxic reactivity. Both of the above mentioned observations involving the continuous culture of normal human and differentiated murine CTLL have been confirmed (4, 5).

We now report the ability to culture large numbers of human antigen-specific cytotoxic T lymphocytes in a TCGF-dependent state of sustained exponential proliferation. This technological advance should allow for the performance of studies that were previously not possible, such as the characterization of cytotoxic T-cell differentiation markers, the molecular mechanism of lymphocyte-mediated cytolysis (LMC), and the nature of the T-cell antigen receptor. In addition, human (H)-CTLL may provide a new tool for the typing of cell surface antigens; as well as provide new insight into the immunotherapeutic manipulation of several disease states, most notably: immunodeficiency, autoimmunity, and the host's response to malignancy.

Materials and Methods

In Vitro Generation and Long-Term Culture of H-CTLL. Antigen-specific cytotoxic T cells were generated in 7 day primary or 10 day secondary one-way mixed lymphocyte culture (MLC). Both responder and stimulator lymphocyte populations were isolated from normal human peripheral blood by Ficoll-Hypaque sedimentation (6) and diluted to 1×10^6 cells/ml in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, Grand Island Biological Co., Grand Island, N. Y., 300 μg/ml L-glutamine, 50 U/ml penicillin-G, 50 μg/ml gentamicin, and 25 μM/ml Hepes buffer (Calbiochem, San Diego, Calif.). Responding lymphocytes (10 ml) were mixed with an equal volume of previously X-irradiated allogeneic stimulator cells and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. After 7 days viable responding lymphocytes were harvested and used as either effector cell populations in 4 h ^{51}Cr-release

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assays or placed into long-term culture. H-CTLL were initiated by culturing MLC effector cells in a solution containing 50% human TCGF and 50% supplemented RPMI-1640.

LMC Assay. H-CTLL were tested periodically for continued expression of stimulator specific cytotoxicity as measured in a 4-h ^{51}Cr-release assay. Targets used were either freshly isolated peripheral blood mononuclear cells or long-term T-cell lines. LMC assays were conducted as previously described (7). Percent specific lysis at varying effector/target cell ratios was calculated according to the following equation:

$$\text{percent specific lysis} = 100 \times \frac{\text{experimental cpm} - \text{medium control cpm}}{\text{maximum release cpm} - \text{medium control cpm}}.$$

Lytic reactivity was detailed in terms of both lytic capacity (LC) and lytic efficiency (LE). LC was defined as the number of lytic units (LU)/10^6 CTLL cells. 1 LU was defined as that number of effector cells required to mediate 30% specific lysis. LE was defined as the percent specific lysis observed at an effector/target cell ratio of 100/1.

Cryopreservation. H-CTLL were periodically cryopreserved with the aid of a Planar programmed slow-freeze apparatus (Planar Co., Sunbury on Thames, England). Cultures were prepared for freezing by resuspension in FCS at 4°C and dilution over a 10-min period with an equal volume of RPMI-1640 supplemented with 20% dimethylsulfoxide and 0.2% EDTA. Frozen human CTLL were maintained in the vapor phase of liquid nitrogen.

Human CTLL Cell Surface Characterization. H-CTLL were tested for their ability to rosette sheep erythrocytes by using methodology previously described (8). H-CTLL were also tested for the cell surface expression of both human T-cell antigen and HLA-D or Ia-like determinants. ^{51}Cr-labeled CTLL cells were used as target cells in antibody-dependent complement-mediated cytolysis assays using absorbed rabbit complement, antigen-specific typing sera, and methodology previously described (7). Both anti-T-cell antigen (A54-5, 10-6) and anti-Ia-like sera (7147-126) were provided by Dr. Jun Minowada, Roswell Park Memorial Institute, Buffalo, N. Y. (9, 10). Positive control human leukemia cells (MOLT-4F and DAUDI or NALM-1, respectively) were also provided by Dr. Minowada (9–11). The cell surface presence of these markers was expressed as the reciprocal of the highest antiserum dilution causing 20% specific lysis.

Results

Cytolytic Reactivity of H-CTLL. H-CTLL-1 was initiated after secondary MLC stimulation ((A + B_X) + B_X) and has remained in continuous TCGF-dependent culture for 4 mo. The continued expression of alloantigen-specific cytolysis by H-CTLL-1 is displayed in Fig. 1. Effector cell populations depicted include H-CTLL-1 cells tested at the conclusion of MLC stimulation and after 6 and 12 wk of culture. H-CTLL-1-mediated, alloantigen-directed cytotoxicity remained remarkably constant over this 3 mo period. It is important to note that self or third-party target cells were not affected thereby confirming that H-CTLL-1-mediated cytolysis was antigen-specific.

Data representing the long-term culture of two additional H-CTLL are shown in Table I. In this experimentation, effector cells were generated in primary 7 day MLC before culture in TCGF-containing medium. The result of LMC assays conducted immediately after MLC and after 4 wk in culture are displayed both in terms of LC and LE. H-CTLL-2 (C + B_X) cells specifically lysed B target cells (410 LU/10^6) effector cells after 4 wk in culture). Similarly H-CTLL-3 (B + C_X) cells were effective in mediating only the lysis of the allogeneic C target cell.

Cryopreservation of H-CTLL. The ability to recover cytolytic T cells from the cryopreserved state would greatly facilitate many of the potential uses of H-CTLL (e.g. tissue typing, immunotherapy). Therefore, experiments were designed in which H-CTLL-1 cells were frozen after 4 wk of culture and maintained in the vapor phase

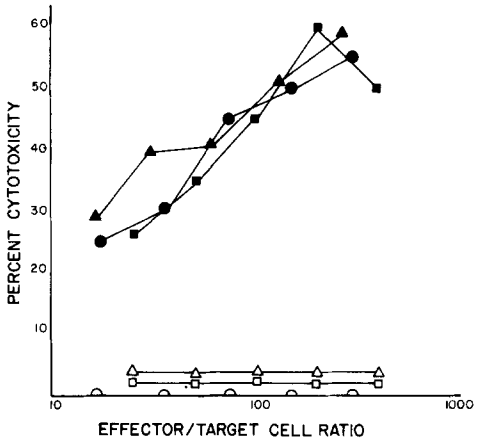

FIG. 1. Cytotoxic reactivity of H-CTLL-1 cells. (A + B_x) + B_x secondary MLC effector-cell-mediated lysis of B target cell (●——●), autologous A target cell (○——○). 6 wk cultured H-CTLL-1 cell-mediated lysis of B target cell (▲——▲), third party C target cell, (△——△). 12 wk cultured H-CTLL-1 cell-mediated lysis of B target cell (■——■), autologous A target cell (□——□).

TABLE I

Cytolytic Specificity of H-CTLL

H-CTLL designation	MLC stimulation	Culture duration	Cytolytic reactivity			
			Target B		Target C	
			Lytic capacity*	Lytic efficiency‡	Lytic capacity	Lytic efficiency
		wk				
H-CTLL-2	C + B_x	0	368	73.9	0.1	1.5
"	"	4	410	85	0.3	8.0
H-CTLL-3	B + C_x	0	<0.1	<1.0	25.0	33
"	"	4	<0.1	<1.0	28.5	38

* Expressed as LU/10^6 CTLL cells.

‡ Expressed as percent specific lysis observed at a CTLL/target cell ratio of 100/1.

of liquid nitrogen. Upon thawing, frozen CTLL were re-established in long-term TCGF-dependent culture. 2 and 4 wk after reinitiation of continuous culture, both previously frozen and control H-CTLL-1 cells were tested for cytotoxic reactivity directed against radiolabeled B target cells. Results of these experiments are detailed in Table II. Both re-established and control cells demonstrated similar high levels of cytolytic reactivity against B target cells.

Cell Surface Characterization of H-CTLL. H-CTLL cells were found to be lymphoblastoid in appearance and 95–100% E-rosette positive. Fig. 2 shows a cytospin preparation of E-rosette positive H-CTLL-1 cells after 11 wk of culture. We also tested H-CTLL-1 cells for the cell membrane expression of two surface markers via antibody-dependent complement-mediated cytolysis assays. The two typing sera used included anti-T-LCL antiserum and anti-Ia-like antiserum. Anti-T-LCL antiserum has previously been shown to contain antibody against a normal T-cell antigen (9). Anti-Ia-like antiserum was raised in rabbits by immunization with the DAUDI human lymphoblastoid cell line. The DAUDI cell membrane is void of HLA antigens A, B,

TABLE II

Stability of H-CTLL Cytolytic Reactivity after Cryopreservation

H-CTLL designation	Culture duration	Cytotoxicity directed against B target	
		Lytic capacity*	Lytic efficiency‡
	wk		
H-CTLL-1	4	118	58
"	6	101	46
"	8	98	42
H-CTLL-1 (frozen 4-wk old cultures)	2 wk after thawing	125	50
H-CTLL-1 (frozen 4-wk old cultures)	4 wk after thawing	112	51

* Expressed as LU/10^6 CTLL cells.

‡ Expressed as percent specific lysis observed at a CTLL/target cell ratio of 100/1.

TABLE III

H-CTLL Cell Surface Characterization

Target cell line	Antibody-dependent complement-mediated cytolysis assay data*	
	Anti-T-cell antiserum‡	Anti-Ia-like antiserum§
CTLL-1	35	20
DAUDI	‖	160
MOLT-4F	40	‖
NALM-1	‖	80

* Expressed as the reciprocal of the highest antiserum dilution which caused 20% specific lysis.

‡ Rabbit anti-T-LCL antiserum (Dr. Jun Minowada, Roswell Park Memorial Institute, Buffalo, N.Y.).

§ Rabbit anti-DAUDI-(HLA-D) antiserum (Dr. Jun Minowada).

‖ No reactivity observed.

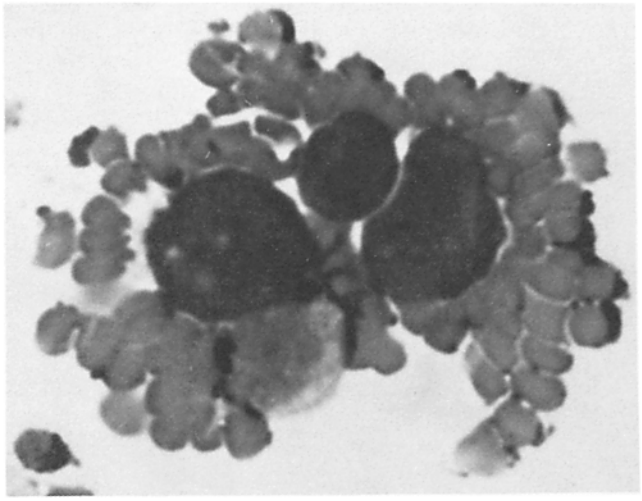

FIG. 2. Cytospin preparation H-CTLL-1 E-rosettes. Magnification ≈ ×5,600.

or C, yet does express an HLA-D encoded glycoprotein (10). The results of the cell surface characterization of H-CTLL-1 cells is detailed in Table III. H-CTLL-1 cells were found to be positive for the surface expression of both normal T-cell and Ia-like antigens. Control cell lines DAUDI, MOLT-4F, and NALM-1 were found to be positive for the respective expression of previously detected cell surface markers (9–11).

Discussion

The ability to sustain antigen-specific human cytolytic T lymphocytes in continuous proliferative culture provides a new technology for the study of cell-mediated immune responses. The cells retain characteristic T-cell surface markers and maintain stable, specific cytolytic activity after prolonged culture or reconstitution from cryopreservation. Such cells will undoubtedly prove useful as tissue typing reagents, for studies on the mechanism of LMC and for characterization of T-cell antigen receptors and T-cell surface markers.

Several of the potential uses for H-CTLL cells listed above would be aided by the

development of monoclonal, antigen-specific CTLL. For example, a battery of cloned H-CTLL, each with a specificity for individual HLA antigens would be invaluable for tissue-typing studies. We have recently been successful in cloning murine CTLL by limiting dilution in microplates.[1] Similar results have also been observed in cloning trials attempted on long-term normal human T-cell lines (F. R. Ruscetti, unpublished observations). Based on these observations it should be possible to develop monoclonal, unifunctional H-CTLL.

One implication of the experimentation described in this report would be the use of tumor-antigen-specific H-CTLL cells for the immunotherapy of human malignancy. We have previously reported the long-term culture of murine tumor-antigen-specific CTLL (3). Murine CTLL maintained in culture for over 20 mo have been found to significantly retard in vivo tumor growth in adoptive transfer experiments.[2] These findings together with recent reports describing in vitro generation of human leukemia-specific cytotoxic cells ([through multi-way mixed tumor lymphocyte stimulation] [12, 13]) suggest that tumor-antigen reactive CTLL might be successful as a new form of specific immunotherapy.

It is important to note that H-CTLL are thoroughly dependent for proliferation upon the presence of TCGF. TCGF deprivation results in H-CTLL cell death within 24–48 h. Recent studies on murine TCGF have shown that: (*a*) T cells are required for TCGF production, (*b*) only T-cell mitogens or antigens elicit TCGF production, and (*c*) only activated T cells absorb TCGF activity (14). These observations provide evidence that a specific TCGF acceptor molecule may exist on activated T cells and that TCGF provides a second signal allowing for the expansion of antigen-activated T-cell clones. Although the immunochemical characterization of TCGF is unknown, a similar B-cell second signal soluble factor (the allogeneic effect factor) has been shown to contain Ia gene region determinants (15). Should TCGF be found to have a similar molecular constituency, a TCGF receptor might help to explain the Ia positive surface characterization of certain murine T-effector cell populations (16) and H-CTLL-1 cells (Table III).

The fact that differentiated antigen-specific T cells can be maintained in continuous culture suggests that similar mechanisms may exist in vivo which operate to control the proliferation of differentiated effector T cells. Investigators may therefore be able to utilize the in vitro system described here to study the regulation of several T-cell immune responses, particularly through the establishment of functional helper or suppressor human T-cell lines. This approach may provide new insight into the regulation of several immunopathological disease states. It is our belief that further study of differentiated T-cell lines and the effects that regulatory molecules (such as TCGF) mediate upon their reactivity will lead to important new discoveries regarding the mechanism of T-cell-mediated immunity.

Summary

Long-term cultures of human cytotoxic T-cell lines (H-CTLL) were established. H-CTLL cells were strictly dependent for growth upon a T-cell growth factor (TCGF) produced by phytohemagglutinin-stimulated normal human peripheral blood lymphocytes. H-CTLL cells were maintained in TCGF-dependent exponential prolifer-

[1] P. E. Baker et al. Manuscript submitted for publication.
[2] S. Gillis et al. Manuscript submitted for publication.

ative culture for over 4 mo during which time they continued to mediate stimulator antigen-specific cytotoxicity as measured by a 4-h ^{51}Cr-release assay. H-CTLL cells recovered from cryopreserved stocks and re-established in long-term culture demonstrated similar high levels of antigen-specific cytotoxicity. H-CTLL cells were 95–100% E-rosette positive and expressed normal T and Ia-like cell surface markers. The ability to sustain differentiated antigen-specific T-effector cells in long-term culture may provide a new means for the study of both the mechanism and regulation of T-cell-mediated immunity.

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