

**MONOCLONAL CYTOLYTIC T-CELL LINES\***

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Recently, there has been considerable interest in the isolation and long-term culture of monospecific cytolytic T lymphocytes. If such cells could be isolated and grown in large numbers, studies could be conducted on the mechanism of T-cell-mediated cytotoxicity, the identification and molecular characterization of the T-cell antigen receptor, and the identification of cytolytic T-cell differentiation markers. We previously reported the successful maintenance of murine cytolytic T cells in sustained exponential proliferative culture with the aid of T-cell growth factor (TCGF) (1). One such cytotoxic T-lymphocyte line (CTLL-2) has remained in culture for 2 yr and has retained both its cytolytic specificity and capacity. We report here the successful creation of cloned monospecific cytolytic T-cell lines from CTLL-2.

**Materials and Methods**

*Derivation of CTLL-2.* CTLL-2 was initiated from spleen cells of a C57Bl/6 mouse (H-2<sup>b</sup>) which had been inoculated with allogeneic Friend leukemia virus (FLV)-induced erythroleukemia cells, F4-5 (H-2<sup>d</sup>). Before initiation of TCGF-dependent culture, a secondary and tertiary in vitro mixed tumor-lymphocyte culture was performed using mitomycin-C inactivated F4-5 stimulator cells (2). Effector T cells harvested from these cultures were found to mediate both allogeneic and syngeneic tumor-specific cytotoxicity (2).

*Long-Term Culture of CTLL-2.* For continuous growth, CTLL-2 cells were seeded at  $1 \times 10^4$  cells/ml in complete Click's medium (Altick Associates, Hudson, Wis.): TCGF, 1:1 (vol/vol). Click's medium was supplemented with 2% fetal calf serum (FCS), 100  $\mu$ g/ml fresh L-glutamine, 50 U/ml penicillin, 50  $\mu$ g/ml gentamicin, 16 mM/ml NaHCO<sub>3</sub>, and 25 mM/ml Hepes Buffer (Calbiochem, San Diego, Calif.). After saturation density was reached ( $5 \times 10^5$  cells/ml) the cultures were reseeded to  $1 \times 10^4$  cells/ml. CTLL-2 has been maintained in culture in this fashion for more than 2 yr. During this period the cells have remained Thy-1 antigen positive and surface immunoglobulin negative.

The production and assay of TCGF were according to previously described methods (3). TCGF used for studies described here was derived from medium conditioned by 48 h concanavalin A ([5  $\mu$ g/ml] [Miles-Yeda Laboratories, Rehovoth, Israel]) stimulation of CD rat (Sprague-Dawley, Charles River Breeding Laboratories, Wilmington, Mass.) spleen cells.

*Creation of Cloned CTLL.* CTLL-2 cells, 14 mo after initiation of culture, were adjusted to 1.2, 1.0, 0.6, and 0.5 cells/ml in complete Click's medium: TCGF, 1:1 (vol/vol), and 250  $\mu$ l was delivered to individual wells of flat-bottomed microtiter plates (3596, Costar, Cambridge, Mass.). After 10–14 d at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air, cells were seeded into 6-well cluster plates (3506, Costar). After reaching a saturation density of  $5 \times 10^5$  cells/ml, cloned cells were seeded into culture flasks (3013, BioQuest, BBL Falcon Products, Cockeysville, Md.) and maintained in a state of TCGF-dependent exponential proliferation.

*Lymphocyte-Mediated Cytotoxicity (LMC) Assay.* FBL-3(Hn) (H-2<sup>b</sup>), P815 (H-2<sup>d</sup>) and F4-5 (H-2<sup>d</sup>)

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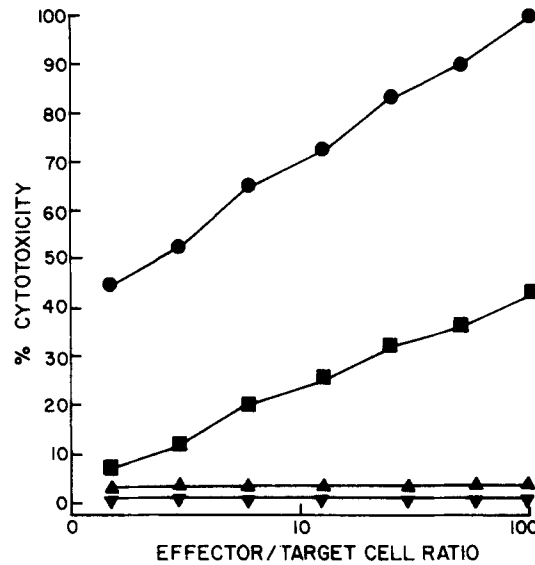


FIG. 1. Cytotoxicity of CTLL-2 against the allogeneic tumor, F4-5 (●), the syngeneic tumor, FBL-3(Hn) (■), normal syngeneic thymus cells (▲), and syngeneic Con A-induced blast cells (▼).

tumor cell lines (2) maintained in RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% heat-inactivated FCS, 300  $\mu\text{g}/\text{ml}$  L-glutamine, 50 U/ml penicillin-G, 50  $\mu\text{g}/\text{ml}$  gentamicin, and  $2.5 \times 10^{-5}$  M 2-mercaptoethanol, were used as targets in 4 h  $^{51}\text{Cr}$ -release assays as previously described (2). In LMC assays performed using cloned effector populations, P815 was chosen as the allogeneic target rather than F4-5 because it allowed the detection of alloantigen-directed cytotoxicity in the absence of antigens associated with FLV-induced transformation.

### Results

The lysis of allogeneic FLV-induced F4-5 leukemia cells and syngeneic FLV-induced FBL-3(Hn) leukemia cells, mediated by CTLL-2 after 17 wk of TCGF-dependent culture is shown in Fig. 1. Although both tumor targets were lysed, normal syngeneic targets were unaffected. Because cytotoxicity of both allogeneic and syngeneic tumor cells occurred, we speculated that CTLL-2 might be composed of at least two different effector cell subpopulations; one with alloantigen specificity, and one with tumor-antigen specificity. To investigate this possibility, CTLL-2 was cloned by limiting dilution as displayed in Table I.

Cells were seeded in microtiter plates at concentrations ranging from 0.30 to 0.13 cells per cloning well. At these cell concentrations, the Poisson calculated probability of any single cloning well populated by only one cell ( $P(K = 1)$ ) was 0.22 to 0.11. The probability of any cloning well being copopulated by more than one cell ( $P(K > 1)$ ) ranged from 0.04 to less than 0.01. The total percentage of calculated fertile wells including those expected to be monoclonal and those expected to be polyclonal was thus 0.26 to 0.12. By comparing these data with the number of fertile wells observed, plating efficiency was found to range from 67 to 100%. This numerical analysis suggests that all clones produced, originated from single cells ( $P < 0.05$ ).

To discern whether the clones displayed monospecific cytotoxic activity, P815 and FBL-3(Hn) tumor cell lines were employed as targets in LMC assays. Each of the 24

TABLE I  
Clonal Isolation of Cytolytic T-Lymphocyte Lines by Limiting Dilution

Clone No.	No. cells/well plated	Poisson probability		No. of wells:			Plating efficiency %
		P (K = 1)*	P (K > 1)‡	Plated	Expected	Grown	
1, 2, 3, 5, 6, 7	0.30	0.22	0.04	24	6	6	100
16, 17, 18, 19, 20 21, 22, 23, 24	0.25	0.20	0.02	46	10	9	90
4, 8	0.15	0.13	0.01	24	3	2	67
9, 10, 11, 12, 13 14, 15	0.13	0.11	0.01	84	10	7	70

\* Poisson calculated probability of one cell occupying any one well.

‡ Poisson calculated probability of more than one cell occupying any one well.

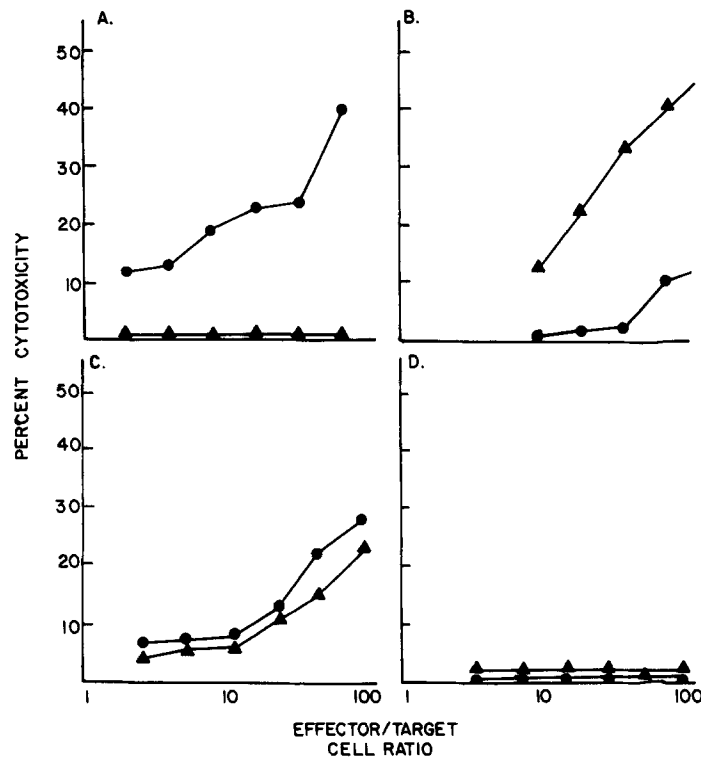


FIG. 2. Representative cytotoxicity of CTLL-2 clones against the allogeneic tumor P815 (●) and the syngeneic tumor FBL-3(Hn) (▲); A, clone 3; B, clone 15; C, clone 24; and D, clone 20.

clones isolated displayed one of four patterns of cytotoxic reactivity, examples of which are graphed in Fig. 2. 10 clones (42%) effectively lysed the allogeneic target cell (P815) as represented by clone 3 (Fig. 2A). Four clones (17%) preferentially lysed the syngeneic tumor target cell as represented by clone 15 (Fig. 2B). Six clones (25%) lysed both allogeneic and syngeneic tumor cell targets with equal efficiency (Fig. 2C, clone 24) and four clones (17%) displayed no cytotoxic reactivity whatsoever (Fig. 2D, clone 20). The data displayed in Fig. 2 therefore confirmed our hypothesis that CTLL-2 contained at least two distinct antigen-specific cytotoxic effector cell populations.

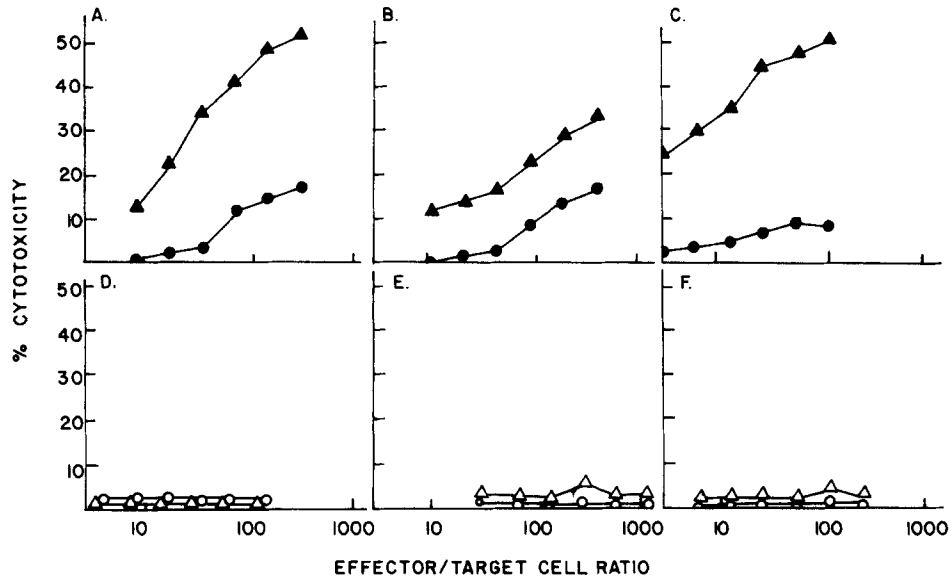


FIG. 3. The stability of clonal cytolytic specificity. Upper panels depict lytic pattern of FBL-3(Hn) (▲) and P815 (●) mediated by clone 15 after: A, 4 wk; B, 8 wk; and C, 13 wk of culture. Lower panels depict lytic pattern of FBL-3(Hn) (Δ) and P815 (□) mediated by clone 20 after: D, 4 wk; E, 7 wk; and F, 8 wk of culture.

To determine whether the pattern of lysis mediated by cloned CTLL remained stable, two clones were selected and tested at successive intervals during several weeks of culture. As shown in Fig. 3A, B, and C, clone 15 consistently mediated the preferential lysis of the syngeneic tumor target cell FBL-3(Hn). Similarly, clone 20 which displayed no cytotoxic activity initially, remained nonreactive during 8 wk of culture, even when tested at effector to target cell ratios as high as 1,000:1 (Fig. 3D, E, and F).

As a final demonstration that cloned CTLL-2 cells were established from single cells, clone 15 was selected and five subclones were created by seeding the cells at limiting dilutions (0.13/cells per well) in microtiter plates. If cloned CTLL were truly derived from a single cell, one would expect to find identical patterns of lysis in all subclones cultured from a single CTLL clone. As shown in Fig. 4, the five subclones of clone 15 displayed lysis patterns identical to the parent CTLL clone.

### Discussion

This experimentation represents the first report of the isolation of monospecific cytolytic T cells and the maintenance of such cells in proliferative culture. Fathman and Hengartner have successfully cloned alloreactive murine T cells; however, the clones lost the capacity to mediate specific cytotoxicity when maintained by repetitive alloantigen stimulation (4). Nabholz et al. attempted to clone alloreactive cytolytic T cells in soft agar, however monospecific cytolytic function was not demonstrated (5). By using the methods described herein, it should now be possible to detail the functional and phenotypic characteristics of differentiated antigen-specific cytolytic T cells. Furthermore, our recent findings regarding the long-term culture of human cytolytic T-cell lines (6) compounded with these results, provide evidence that it

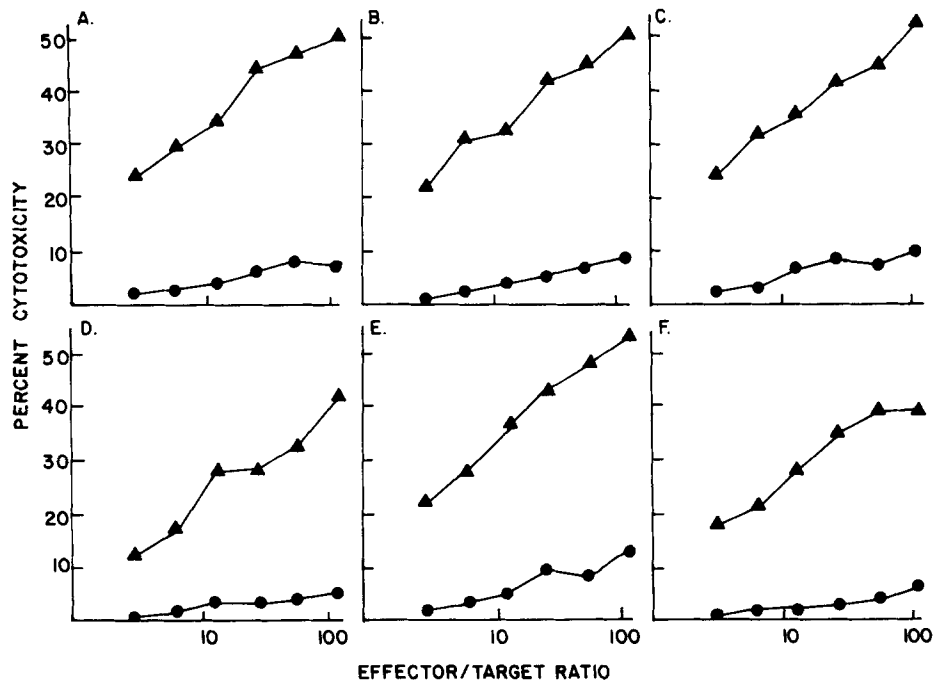


FIG. 4. Cytotoxicity of clone 15 and subclones against FBL-3(Hn) (▲) and P815 (●) 6 wk after isolation of the subclones; A, clone 15; B, subclone 15-1; C, subclone 15-2; D, subclone 15-3; E, subclone 15-4; and F, subclone 15-5.

should be possible to create monospecific cytolytic human T-cell lines.

It is of interest that greater than 80% of the clones derived from CTLL-2 expressed cytolytic function. One would not necessarily have predicted such a high frequency of cytolytic clones in light of recently reported evidence that precursors of cytotoxic cells comprise less than 10% of splenic T cells (7). It is possible that the tertiary allogeneic MTLC used to derive CTLL-2 effector cells in some way selected for cytotoxic cells. Alternatively, TCGF may preferentially act on cytolytic cells rather than helper or suppressor T cells. In other experimentation we have found that only mitogen or antigen-activated T cells absorb TCGF activity<sup>1</sup> suggesting that activated T cells express a TCGF receptor. Our cloning data would suggest therefore, that cytotoxic T cells may express receptors with a higher TCGF affinity than helper or suppressor T cells.

Although the exact biochemical nature of TCGF remains to be elucidated, our previous studies (3, 8) indicate that TCGF is the second signal in the T-cell immune response and that it functions to mediate the proliferative expansion of antigen-activated T-cell clones. The observation that it is possible to select and clone cytolytic T cells in vitro using TCGF, coupled with the ability to maintain such clones in an apparent indefinite TCGF-dependent proliferative state, should greatly facilitate future studies of differentiated cytolytic T cells.

### Summary

Monospecific cloned cytolytic T-lymphocyte lines have been created utilizing T-

<sup>1</sup> K. A. Smith et al. Manuscript in preparation.

cell growth factor. The clones were found to retain their cytolytic specificity after prolonged culture and monospecific function was demonstrated by subcloning procedures. Thus, detailed studies of the phenotypic and functional characteristics of monospecific, homogeneous, cytolytic T lymphocytes will now be possible.

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