

Long term culture of tumour-specific cytotoxic T cells

MANY investigators have been successful in the maintenance of long term tissue culture of human bone marrow-derived (B) cells. These cell lines have been established from both normal subjects¹ and from patients with lymphoproliferative disorders². In most cases, long term B-cell lines have been shown to harbour the Epstein-Barr virus genome which some investigators feel is required for establishment and maintenance of long-term cultures³. There are fewer reports describing continuous culture of human thymus derived (T) cell lines, and when successful, the lines have only been established from patients with acute lymphocytic leukaemia⁴. Although these cell lines have been shown to bear surface markers of normal human T lymphocytes, there have been no reports which suggest that they possess the ability to respond to immunologic stimuli or to differentiate into antigen-specific lymphocytes. Cytotoxic murine T cells have been kept in continuous culture only through repetitive mixed-lymphocyte stimulation⁵. In contrast to long term human lymphocyte lines, these cells proliferated only when stimulated with allogeneic lymphocytes and eventually died after a few weeks in culture. Morgan, Ruscetti and Gallo recently reported a method by which medium conditioned by phytohaemagglutinin-stimulated normal human lymphocytes allowed for the selective long-term growth of normal T cells⁶. In contrast to the previously mentioned cell lines, the proliferation of these reported T-cell cultures was totally dependent on the presence of an exogenously-produced growth factor supplied by the conditioned medium. In this report we describe an adaptation of this method which allows the long term culture of antigen-selected cytotoxic T cells which continue to demonstrate high levels of syngeneic tumour-specific cytotoxicity after more than 4 months in culture.

Cytotoxic T cells were generated in secondary allogeneic mixed tumour-lymphocyte cultures (MTLC). Spleen cells were harvested from normal C57B1/6 mice and from C57B1/6 mice immunised with allogeneic (*H-2^d*) F4-5 Friend virus (FLV)-induced leukaemia cells (from Dr W. Ostertag, Max Planck Institute, Göttingen, Germany). Both spleen cell popu-

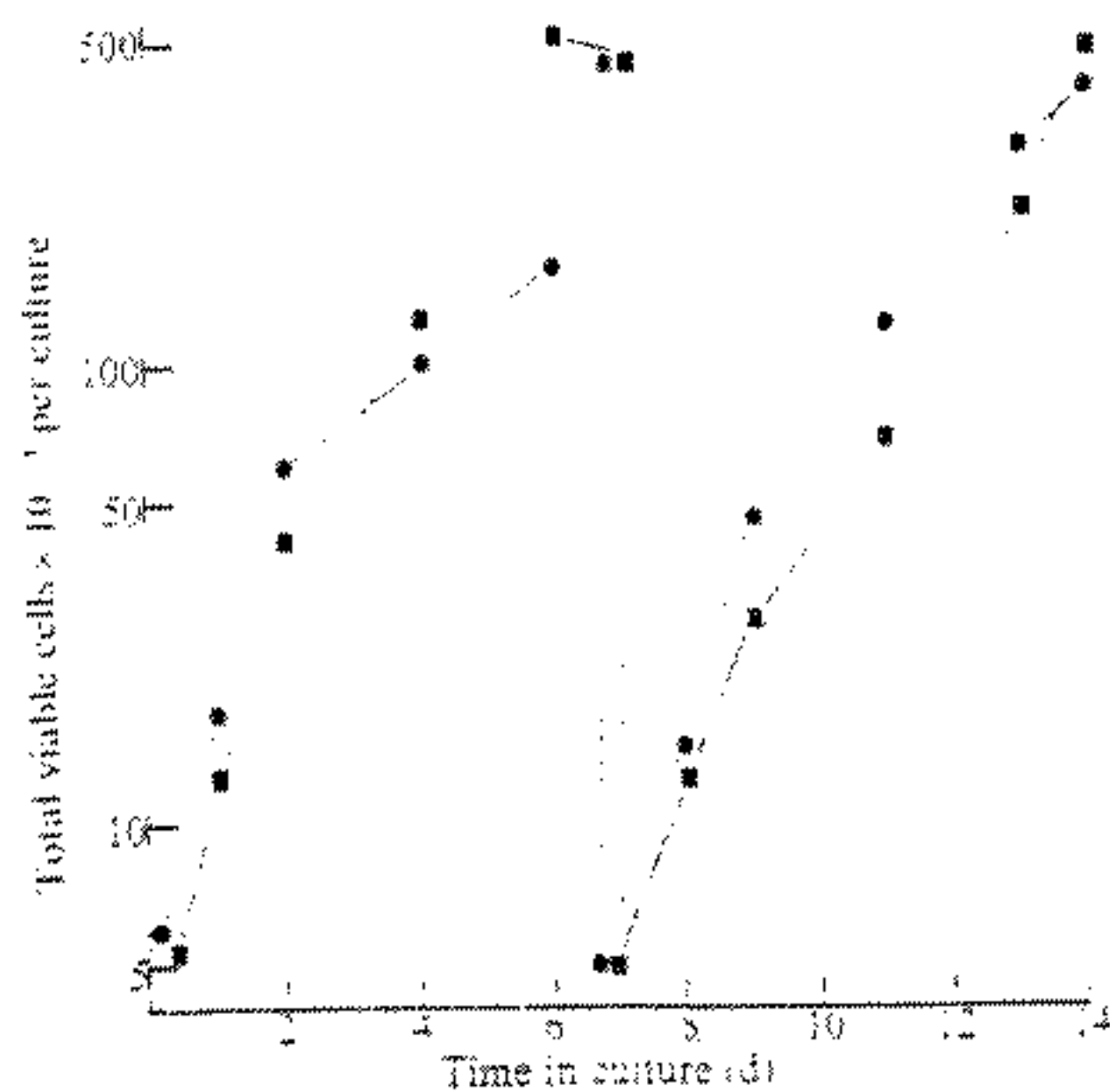


Fig. 1 Representative growth curves of CTLL(1) (●) and CTLL(2) (■). Dotted lines indicate passage of long term cell lines and re-seeding of additional culture flasks at the day 0 cell concentration of 5×10^3 cells per ml. Both cell lines exhibited this pattern of proliferative growth provided they were grown in the presence of medium containing 50% growth factor. Click's medium with 50% growth factor was also supplemented with 2% heat-inactivated FCS, 300 $\mu\text{g ml}^{-1}$ fresh L-glutamine, 50 units ml^{-1} penicillin, 50 $\mu\text{g ml}^{-1}$ streptomycin, 16 $\mu\text{mol ml}^{-1}$ NaHCO_3 and 25 $\mu\text{mol ml}^{-1}$ HEPES buffer. Cells were cultured in a humidified atmosphere of 5% CO_2 in air at 37 °C.

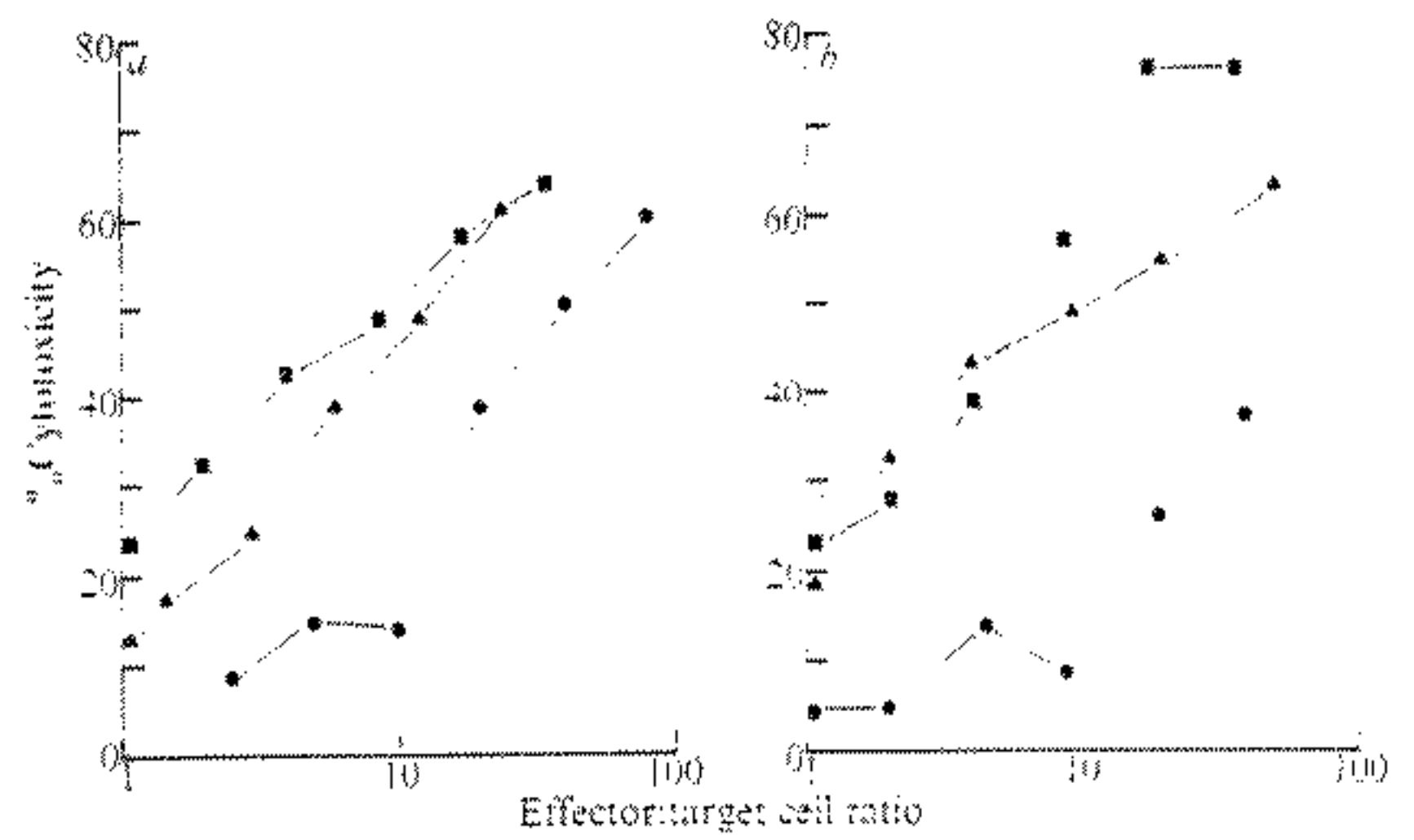


Fig. 2 *a*, Allogeneic cytotoxicity demonstrated by CTLL(1) against the FLV-induced non-producer murine leukaemia cell, F4-5, after 3 weeks (●), after 10 weeks (▲), and after 17 weeks (■), in continuous culture. *b*, Cytotoxicity demonstrated by CTLL(2) against F4-5 after 3 weeks (●), 10 (▲) and 17 weeks (■) in culture. Per cent cytotoxicity was determined in lymphocyte-mediated cytotoxicity assays which measured Cr^{51} release of radiolabelled target cells. Target cells were labelled with 250 μCi of $\text{Na}_2^{51}\text{CrO}_4$ (specific activity 100–400 mCi per mg Cr , Amersham-Searle). Target cell concentrations were adjusted in RPMI 1640 with 10% FCS to 0.1 c.p.m. per cell. Target cells (100 μl) were mixed with log₂ dilutions of effector cells collected from CTLL(1) and CTLL(2) long term cultures, in V-bottom microplates (15 MVC-96-TC, Linbro Scientific). The Cr-release reaction was stopped after a 4-h incubation at 37 °C by a 10-min 300g centrifugation at 4 °C. c.p.m. of Cr-release were determined by counting 100 μl of each well supernatant on a liquid scintillation counter, per cent specific lysis was determined using the following equation

$$\% \text{Specific lysis} = \frac{\text{experimental c.p.m.} - \text{medium control c.p.m.}}{\text{maximum release c.p.m.} - \text{medium control c.p.m.}} \times 100$$

Medium control c.p.m. were obtained from 100- μl supernatant aliquots sampled from microplate wells containing 100 μl of target cells and 100 μl of RPMI 1640 with 10% FCS. Maximum release c.p.m. were generated from microplate wells containing 100 μl of target cells and 100 μl of Zap Isoton Solution (three drops added to 5 ml double distilled H_2O), Coulter Electronics. Lytic units were defined as the number of effector cells necessary to cause 30% specific lysis. Growth factor was not present in LMC assays.

lations were co-cultured with mitomycin-treated ($100 \mu\text{g ml}^{-1}$ for 60 min)⁷ F4-5 cells in Click's medium (Altick Associates) with 2% heat inactivated foetal calf serum (FCS) for 5 d (responder: stimulator ratio of 40:1). Viable cells collected from primary allogeneic MTLC were restimulated with mitomycin-treated F4-5 cells and placed back in culture for a further 2 d. Cultures of cytotoxic cells generated in secondary allogeneic MTLC of normal C57B1/6 spleen cells were designated cytotoxic lymphoid line 1 [(CTLL(1))] and cells collected from repetitive allogeneic MTLC of spleen cells from C57B1/6 mice immunised with F4-5 were designated CTLL(2). The population of cytotoxic lymphocytes collected from secondary allogeneic MTLC was a heterogeneous one in that one lymphoid subpopulation was reactive against allogeneic targets while a second subpopulation contained lymphocytes capable of effecting syngeneic tumour-specific cytotoxicity^{8,9}.

Viable cells collected from secondary allogeneic MTLC were placed in continuous culture in 50% Click's medium and 50% growth factor. Growth factor was prepared from 48-h tissue culture medium (RPMI 1640 (GIBCO) with 10% FCS) from cultures of concanavalin A (con A, 2.5 $\mu\text{g ml}^{-1}$, Miles-Yeda) stimulated normal DBA.2 spleen cells. Growth factor was filtered through a 0.2- μm filter (Nalge-Sybron) to remove subcellular particles before use in long-term culture medium.

Both CTLL(1) and CTLL(2) have remained in continuous proliferative culture for 22 weeks. Representative growth

curves for both lines are shown in Fig. 1. Cells seeded at a concentration of 5×10^3 per ml reach a saturation density of approximately 3×10^6 cells per ml after 6 or 7 d in culture thereby demonstrating a doubling time of 24 h. Proliferative growth is totally dependent on the presence of growth factor. CTLL(1) and CTLL(2) cannot be maintained in continuous culture in either Click's medium supplemented with FCS or in RPMI 1640 medium supplemented with FCS and con A.

Cells from cultures of CTLL(1) and CTLL(2) were used periodically as effector cells in lymphocyte-mediated cytotoxicity (LMC) assays⁸, against the allogeneic tumour target cell, F4-5 (Fig. 2) and the syngeneic FLV-induced FBL-3 (Hn), (Fig. 3). Both F4-5 and FBL-3 (Hn) were confirmed as non-virus-producing murine leukaemia cells by reverse transcriptase⁹ and X-C assays¹⁰. When LMC data is expressed in terms of lytic units per 10^6 effector cells ($LU \times 10^{-6}$), the allogeneic cytotoxicity mediated by CTLL(1) increased from $76.9 LU \times 10^{-6}$ after 3 weeks in culture to $555 LU \times 10^{-6}$ after 17 weeks in culture. Similarly, allogeneic lysis effected by CTLL(2) rose from $40 LU \times 10^{-6}$ after 3 weeks of culture to $500 LU \times 10^{-6}$ 14 weeks later. Results of LMC assays (Fig. 3) indicate that the syngeneic tumour cell lysis mediated by CTLL(1) and CTLL(2) was tumour specific. Although FBL-3 (Hn) murine leukaemia cells were effectively lysed, syngeneic normal cells (C57B1/6 lymph node and con A-stimulated ($2.5 \mu\text{g ml}^{-1}$) spleen cells remained unaffected). Syngeneic tumour-specific cytotoxicity demonstrated by CTLL(1) effector cells increased from $10 LU \times 10^{-6}$ after 3 weeks in culture to $333 LU \times 10^{-6}$ after 17 weeks in culture. CTLL(2)-mediated cytotoxicity of FBL-3 (Hn) cells rose from $16.7 LU \times 10^{-6}$ following 3 weeks in culture to $142.9 LU \times 10^{-6}$ after 17 weeks of continuous *in vitro* passage.

Figure 4 shows Wright-Giemsa-stained preparations of CTLL(1) and CTLL(2) after 22 weeks of continuous proliferation *in vitro*. Both cell lines are blastoid and highly vacuolar in appearance, and were lysed by anti-theta serum (AKR anti-C3H thymus from Litton Bionetic) when tested using absorbed rabbit complement¹¹. Histochemical stains of both CTLL(1) and CTLL(2) for peroxidase, periodic acid Schiff, naphthal chloroacetate and α -naphthylacetate esterases, were negative. These tests confirm that the syngeneic tumour-specific cytotoxicity demonstrated by CTLL(1) and CTLL(2) was mediated by cytotoxic T cells maintained *in vitro* with the aid of a growth factor produced by con A-stimulated spleen cells.

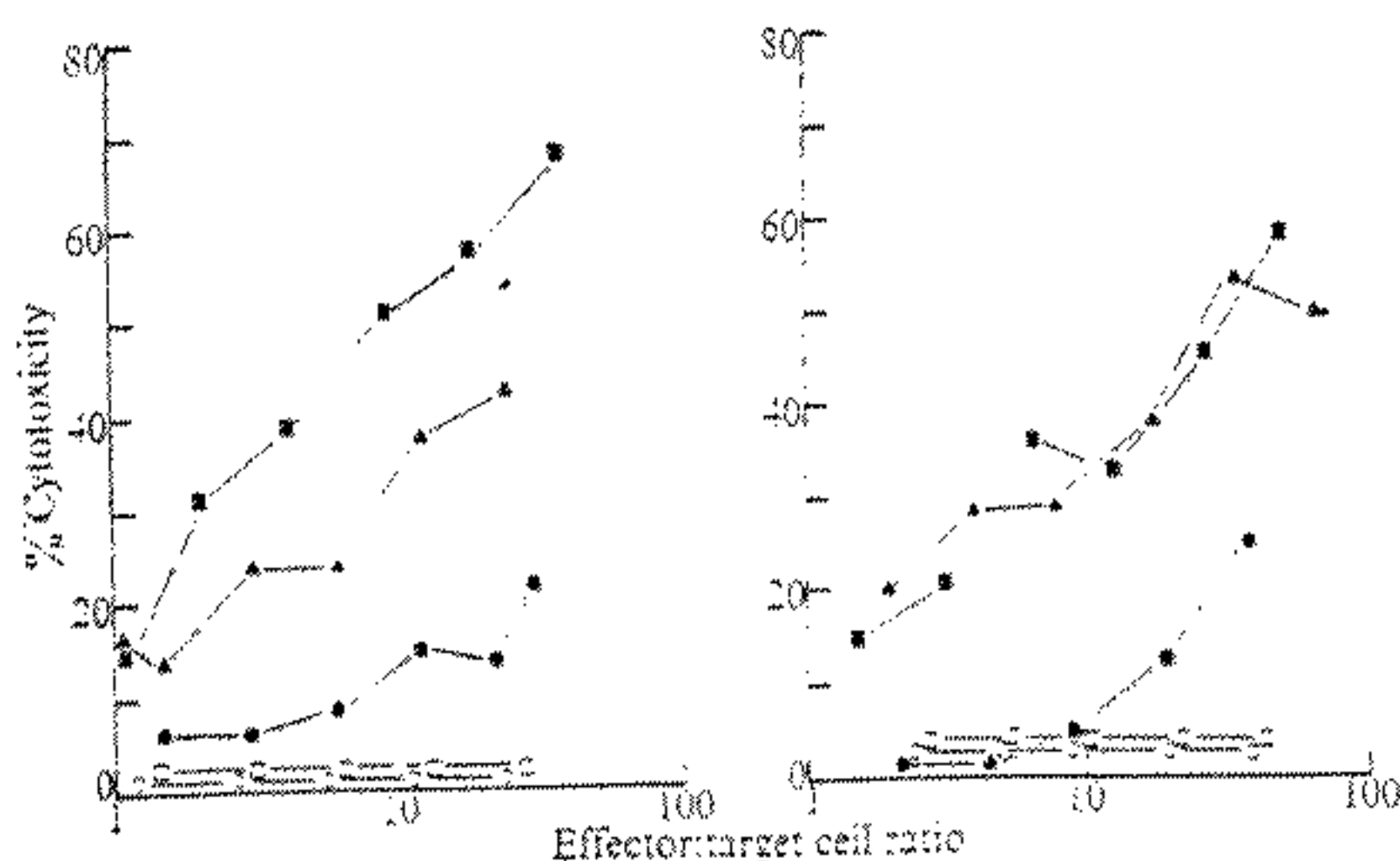


Fig. 3 a, Cytotoxicity of syngeneic FBL-3 (Hn) Friend leukaemia cells effected by CTLL(1) cells after 3 (●), 10 (▲) and 17 weeks (■) in culture. Cytotoxicity demonstrated by CTLL(1) against syngeneic normal lymph node (○) and con A-stimulated spleen cells (□). b, Cytotoxicity of FBL-3 (Hn) by CTLL(2) cells after 3 (●), 10 (▲), and 17 weeks (■) in culture. CTLL(2)-mediated cytotoxicity of syngeneic normal lymph node (○) and con A-stimulated spleen cells (□). FBL-3 (Hn) was adapted to continuous *in vitro* culture from ascites-passaged FBL-3 cells, obtained from Dr Ronald Herberman, NCI. LMC assay conditions, and radiolabelling of all target cells identical to those described in Fig. 2.

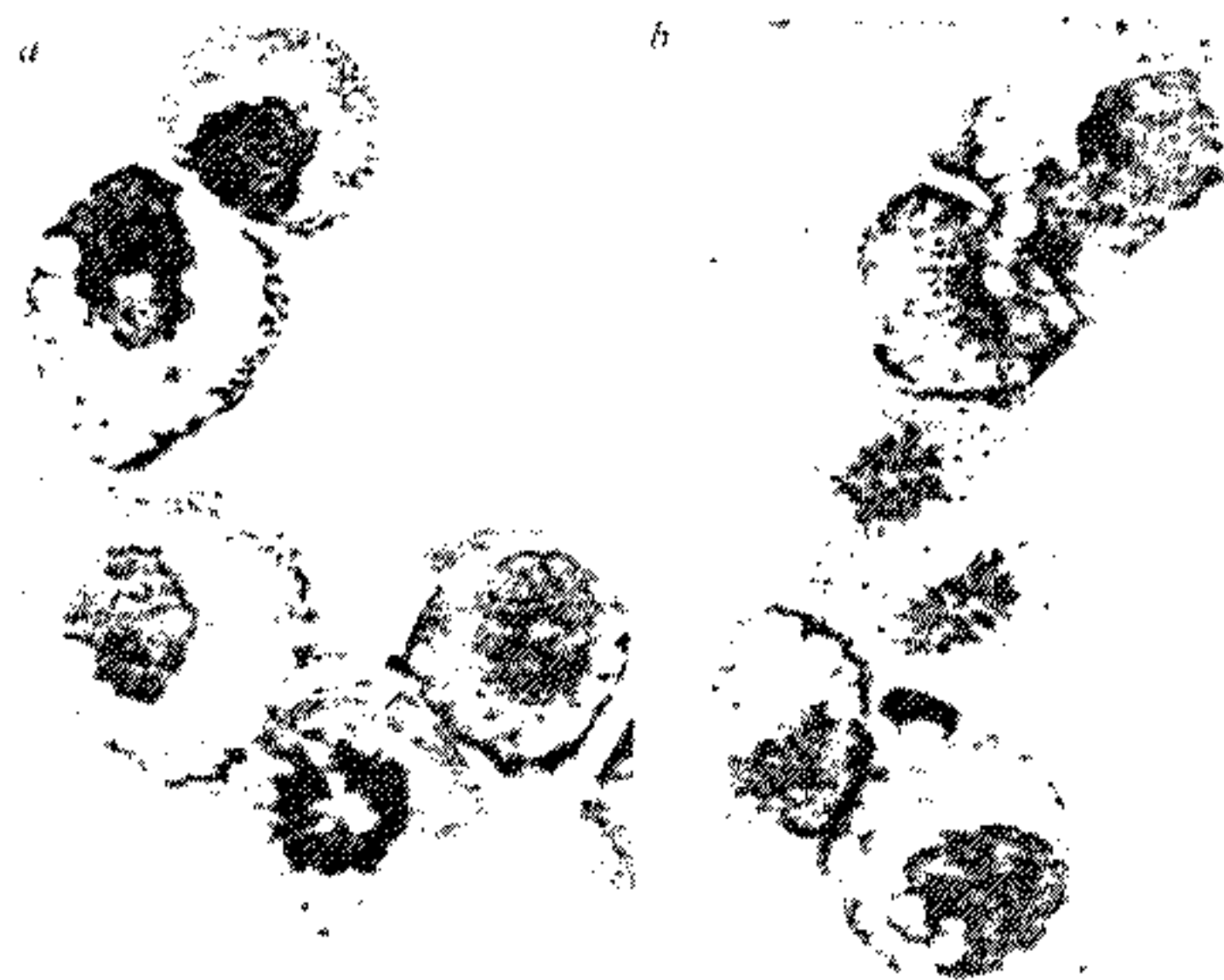


Fig. 4. Wright-Giemsa-stained cytocentrifuge preparations of a, CTLL(1) and b, CTLL(2) cells after 22 weeks of continuous culture *in vitro*. ($\times 6,000$).

The continuous proliferation of differentiated T lymphocytes *in vitro* raises questions as to the regulatory factors operating *in vivo* which prevent continuous clonal expansion after antigenic stimulation. The demonstration that continuous proliferation of cytotoxic T cells *in vitro* is possible, suggests that some humoral or cellular factor may be operating *in vivo* to prevent differentiated proliferation. Furthermore, the long term culture system described here might serve as a starting point for the identification and characterisation of these growth promoting and inhibiting, regulatory factors.

Finally, although cytotoxic lymphocytes generated *in vitro* have been shown to be effective mediators of tumour cell lysis *in vivo*¹², the inability to generate these cells in large numbers has prevented their widespread use. The ability to propagate large populations of syngeneic tumor antigen-specific cytotoxic lymphocytes through the use of a growth factor, makes possible the testing of these lymphocytes in adoptive immunotherapy. The syngeneic cytotoxic specificities of the long-term T-cell lines tested in this study were directed against non-producer leukaemia cells. Because murine non-producer leukaemia cells represent the model system most analogous to human leukaemia, this study provides evidence that the *in vitro* generation of large numbers of leukaemia-specific human cytotoxic T cells might be possible.

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