

Activated lymphocyte killer cells derived from melanoma tissue or peripheral blood

G. F. BURNS,* M. F. GOOD,† CLARE RIGLAR,* P. F. BARTLETT,† R. M. CRAPPER* & I. R. MACKAY** *Lions Clinical Cancer Laboratory, Clinical Research Unit of The Walter and Eliza Hall Institute of Medical Research and Royal Melbourne Hospital and*
† *Cellular Immunology Unit, The Walter and Eliza Hall Institute, Victoria, Australia*

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SUMMARY

Lymphoid cells infiltrating metastatic melanomas were grown directly from cell suspensions of tumour tissue by the addition of T cell growth factor. Lymphoid cells grew out at the expense of tumour cells in six of seven freshly excised tumours, and cells from two cultures were expanded for *in vitro* testing of cytolytic function against different target cells. Early in culture the tumour derived lymphocytes killed fresh autologous melanoma cells and, particularly later in culture, were highly and non-specifically cytolytic for cultured melanoma and non-melanoma cells. Cultured peripheral blood lymphocytes from patients with melanoma, and from normal subjects, were cytolytic to the same degree as tumour derived lymphocytes, and also resembled cells grown from tumour tissue in possessing acid phosphatase activity which was resistant to tartrate. Cultured lymphoblasts from both tumour and peripheral blood had a T cell phenotype when analysed with monoclonal antibodies. An *in vitro* co-culture system was employed to study the kinetics and the precursors of these non-specific killer cells among blood mononuclear cells. Blood mononuclear cells cultured with irradiated B lymphoblasts led to the generation of non-specific cytolytic cells, referred to as activated lymphocyte killer (ALK) cells, after 7–10 days of culture and the progenitors of these ALK cells were demonstrated to be distinct from those of specific cytolytic T cells

Keywords activated lymphocyte killer melanoma

INTRODUCTION

Histological examination of human tumours frequently reveals the presence of infiltrating lymphoid cells and, in the case of melanoma, a high degree of lymphoid infiltration is associated with tumours which regress spontaneously (Underwood, 1974). However the lymphocytes found within solid tumours in man have been relatively little studied due in part to difficulty in obtaining good yields of purified viable cells, and functional studies employing tumour derived lymphocytes have been few (Vose, Vánky & Klein, 1977; Tötterman *et al.*, 1978).

Recently lymphocytes with a T cell phenotype have been grown from cell suspensions prepared from freshly resected breast cancers and melanomas, and the number of T cell colonies in agar culture correlated with histological assessment of the degree of lymphocytic infiltration within the tumour (Asano *et al.*, 1981). In the present study we set out to characterize the lymphocytes cultured

from excised melanomas and to compare their phenotype and cytolytic activity with that of lymphoblasts cultured from peripheral blood using T cell growth factor (TCGF).

The properties of the cytolytic lymphoblasts thus obtained proved to be neither those of natural killer (NK) cells nor classical cytolytic T cells (CTC). We considered these cells to be activated lymphocyte killer (ALK) cells and, in view of their ability to kill melanoma cells rapidly, they may function *in vivo* to regulate tumour cell growth.

MATERIALS AND METHODS

Patients. Seven tumours were excised from six patients; one patient (IW) had two separate surgical excisions. The metastatic tumours excised were from lymph nodes (IW2, GW), subcutaneous tissue (DA and LT), brain (SG) and skin (DN). In all cases the presence of melanoma was confirmed by histological examination. The actual percentage of lymphocytes present in the tumours was not quantitatively assessed but in each case melanoma cells greatly outnumbered lymphocytes.

Treatment of melanoma tissue. Portions of freshly resected tumours were collected into cold Dulbecco's modified Eagle's medium (DME) and were disrupted mechanically to a single cell suspension as described previously (Asano & Riglar, 1981). Specimens with a high ratio of erythrocytes to nucleated cells were discarded to reduce the possibility that cultured T cells could be derived from blood lymphocytes, and dead cells were removed by centrifugation over Ficoll-Hypaque. For growth of tumour cells, the cells were washed in DME + 20% fetal calf serum (FCS), resuspended to 5×10^5 cells/ml and cultured in the same medium. Freshly isolated tumour cells were also frozen in liquid nitrogen.

Lymphocytes were grown directly from tumour tissue by adding PHA-lymphocyte conditioned medium (CM) (see below) as a source of TCGF. The single cell suspension was washed in RPMI 1640 medium containing 20 mM HEPES, 10% FCS (Flow), 5×10^{-5} M 2-mercaptoethanol, and antibiotics (complete RPMI), and cultured at 1×10^6 cells/ml in the same medium containing 10% CM, in tissue culture microplates (Linbro 76-013-05), 24 well trays (Linbro 76-033-05) or culture flasks (Falcon 5375).

Induction of specific CTC from peripheral blood cells. B lymphoblasts transformed with Epstein-Barr virus were established from blood mononuclear cells of normal subjects and specific CTC against irradiated autologous B lymphoblasts were then induced by co-culture (Burns, Boyd & Beverley, 1982b).

Preparation of CM. CM containing TCGF was prepared from leucocytes obtained from venesection of patients with haemochromatosis, and the leucocytes were stimulated with 1% phytohaemagglutinin (PHA) (Wellcome Laboratories) (Burns, Battye & Goldstein, 1982a). Residual PHA was removed by passage over a thyroglobulin-Sepharose column (Burns *et al.*, 1982a). The level of interferon (IFN) in the CM was measured by a virus plaque inhibition assay by courtesy of Dr J. Breschkin, and each batch of contained between 50–100 units of activity.

Cytolytic assay. Cytolytic activity was routinely assessed with a standard 4 h ^{51}Cr release assay at an effector:target ratio of 10:1 (Burns *et al.*, 1982b), unless stated otherwise in Results.

Target cells. The two melanoma cell lines used, LiBr and TAM, were established in this laboratory from resected skin melanomas. Other targets included Chang cells, K-562 cells and cells from the clones of B lymphoblasts cells used for stimulation. These cell lines were maintained in DME containing 10% FCS, penicillin and streptomycin and were demonstrated to be clear of mycoplasma.

Mouse monoclonal antibodies. Antibodies of the Leu series (Leu 2a and Leu 7) were obtained from Becton Dickinson & Co. (Sunnyvale, California, USA); OKT3, OKT4 and OKT8 from Dr G. Goldstein (Ortho Pharmaceutical Co., Raritan, New Jersey, USA); 3A1 from Dr B.F. Haynes (Duke University, North Carolina, USA) and Dr A.S. Fauci (NIH, Bethesda, Maryland, USA); UCHT1 (equivalent to OKT3) from Dr P.C.L. Beverley (ICRF, University College Hospital, London, UK); FMC17 which reacts strongly with monocytes and weakly with granulocytes from

Drs D.A. Brooks and H. Zola (Flinders Medical Centre, South Australia); and A2 against the sheep erythrocyte receptor from Dr I.F.C. McKenzie (University of Melbourne, Victoria).

Cytochemistry. Standard cytochemical methods were employed for the detection of periodic acid-Schiff (PAS), acid phosphatase with and without L-tartrate, non-specific esterase, sudan black, peroxidase, AS-D chloroacetate and alkaline phosphate, on cytocentrifuge preparations as described previously (Hayhoe *et al.*, 1978).

Cell staining and flow cytometric analysis. Cultured cells were stained by direct or indirect immunofluorescence and analysed by flow cytometry (FACS II, Becton Dickinson FACS Systems, California, USA) as described by Burns *et al.* (1982a).

RESULTS

Growth of lymphoblasts from tumour tissue

Observations on the outgrowth of T cells from melanoma cell suspensions were conducted with cells distributed into the wells of 24 well trays. In the presence of CM the monodispersed cells from the tumour spread out and began to divide slowly. For 4–5 days these cultures resembled the tumour cell cultures without CM, but thereafter there emerged small clusters of cells identified as lymphoblasts; within 8 days these totally replaced the tumour cell monolayer. The emergent lymphoblasts continued to proliferate rapidly for several weeks, provided CM was supplemented every 3 days. A similar sequence of events was observed for six of seven tumours successfully cultured in this way, irrespective of the source of tissue. With one of the seven tumours (DN), a small nodule from a skin metastasis, CM was added but no lymphoblasts grew out even after 2 weeks of culture, although the tumour cells remained viable and divided at the same rate as those in control cultures not receiving CM; lymphocytes were not seen histologically in this tumour.

After varying periods in culture, lymphoblasts obtained from within the different tumours had proliferated to 10^{7-8} cells but then, even with regular feeding with CM, the cells ceased to divide and began to die out.

Cytology and cytochemistry of melanoma derived lymphoblasts

Morphologically the cells growing from melanoma tissue were identical to cells grown from peripheral blood or bone marrow with CM, appearing as large lymphoblasts with a high nuclear/cytoplasmic ratio, numerous mitotic figures, and with a deep blue cytoplasm when stained with Romanowsky stains. The cytochemical profile of the cells was that of lymphoid blast cells; the most striking cytochemical feature was strong acid phosphatase activity in the cytoplasmic granules. This acid phosphatase enzyme, which became less prominent with time in culture, was demonstrated to be resistant to L-tartrate (Fig. 1).

Surface markers of melanoma derived lymphoblasts

The lymphoblasts cultured from all of the tumour specimens formed strong spontaneous rosettes with sheep erythrocytes. Lymphocytes cultured from patient IW for 6 and 28 days were also tested by indirect immunofluorescence with a panel of monoclonal antibodies and analysed by flow cytometry (Fig. 2). These cultured lymphoblasts expressed a T cell phenotype. In additional experiments (not illustrated) 90% of the cells were OKT 3, UCHT 1, 3A1 and A2 positive, predominantly OKT8 positive (56%) but Leu 7 and FMC 17 negative. Peripheral blood lymphocytes from one patient with melanoma and from 10 normal subjects cultured in CM or activated in mixed cell culture for similar periods of time showed a similar surface marker profile.

Cytolytic activity of melanoma derived lymphoblasts

Lymphoid cells grown from two melanomas (IW and LT) were expanded in liquid culture and tested for cytotoxic activity against autologous tumour cells, unrelated melanoma cell lines and Chang cells. Lymphocytes from both of the tumours studied had a high level of cytolytic activity at low E:T ratios against autologous tumour cells, and minimal activity against autologous B lymphoblasts (Table 1). However, particularly after longer periods of culture, the tumour derived

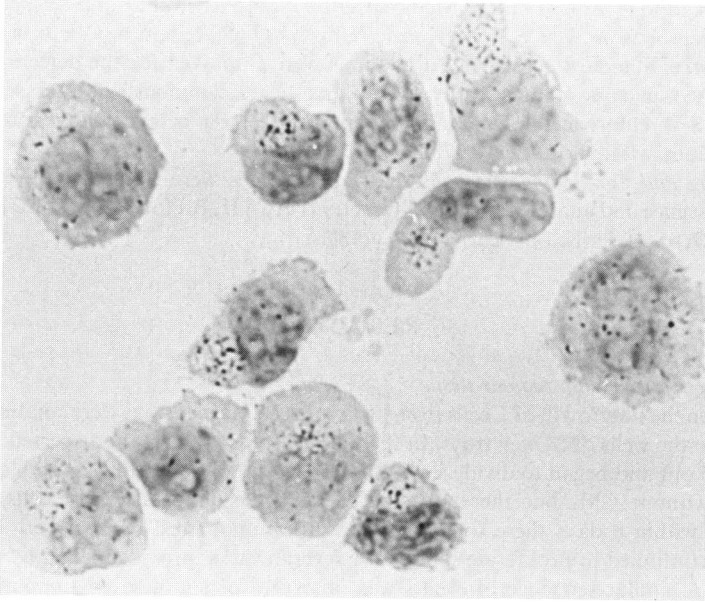


Fig. 1. Tartrate resistant acid phosphatase activity of cultured ALK cells. After some 2 weeks in culture the cells are homogeneous in size and display moderate granular tartrate resistant acid phosphatase activity.

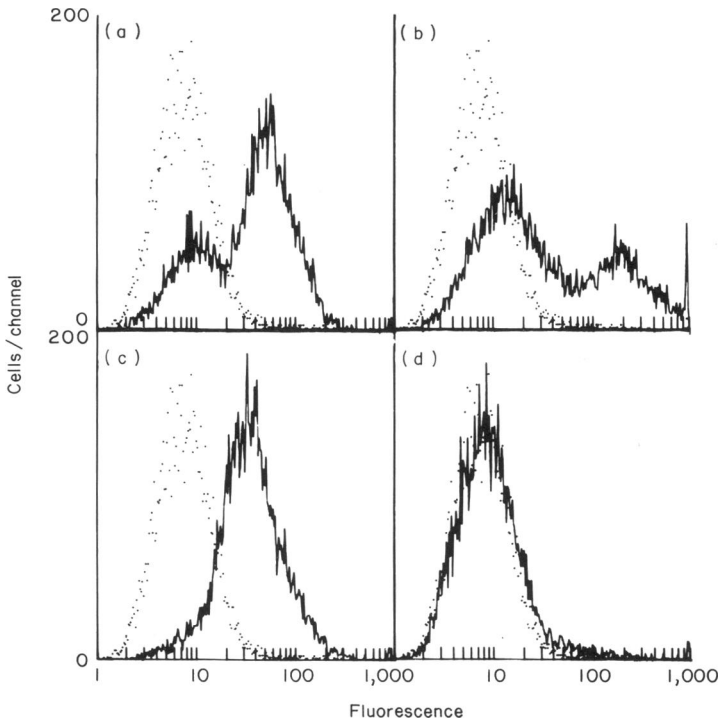


Fig. 2. Surface marker phenotype of the lymphoblastoid cells grown from tumour tissue with CM and analysed by flow cytometry. After 28 days of culture the cells were harvested and stained by direct (Leu 2a, Leu 7) or indirect immunofluorescence. (a), (b) and (c) show that most cells are positive for the T cell markers UCHL1, A2 and Leu 2a, and (d) shows the absence of cells binding Leu 7. In each panel the dotted line is staining with FITC labelled anti-mouse Ig antibody alone; the fluorescent intensity on the ordinate is shown as a logarithmic scale.

Table 1. Cytolytic activity of killer cells generated from peripheral blood (PB) and tumour tissue

Source of killer cells	Days in culture	Mean specific lysis (%) against target				
		Freshly isolated melanoma cells	Melanoma cell lines		Chang cells	Eptstein-Barr virus infected B lymphoblasts
			TAM	LiBr		
Patient LT (tumour)	18	28†	8	ND*	6	6†
Patient IW (tumour)	6	45†	24	29	38	8†
Patient IW (tumour)	8	50†	ND	ND	50	11†
Patient IW (tumour)	21	69†	34	62	62	15†
Patient LT (PB)	12	19†	18	60	55	3
Patient IW (PB)	21	62†	25	70	54	11†
Normal 1 (PB)	14	81	41	81	75	60
Normal 2 (PB)	21	49	ND	ND	25	11

* ND = not done; † autologous target cells.

cytotoxic lymphocytes killed tumour cell targets from various sources, so that their killing was not restricted by MHC antigens.

Cytolytic lymphoblasts from peripheral blood cells

Since cells similar to those derived from melanoma tissue could be grown from peripheral blood it was important to show that the cytolytic cells from tumour tissue were not demonstrable merely by reason of blood contamination. Blood was collected from two patients when the tumour was resected and cultured concurrently with the tumour cells; immediately upon isolation natural killer (NK) activity was <4% at an E:T target ratio of 10:1, and <5% at a ratio of 100:1 against autologous tumour cells. After culture for 6 days, alone in CM or co-cultured with 2,000 rad irradiated autologous tumour cells, these cells exhibited very low levels of cytolytic activity compared with the lymphoblasts grown directly from within the tumour tissue. However, after longer periods of culture in CM, the peripheral blood cells of patients, and of normal subjects, expressed high levels of cytolytic activity at low E:T ratios against a range of tumour target cells (Table 1).

Hence further experiments were done to characterize the kinetics of the appearance of cytolytic lymphoblasts from blood mononuclear cells co-cultured with autologous B lymphoblasts. The products of the co-culture were examined daily for cytolytic activity against the inducing B lymphoblasts as a measure of specific CTC, and against K-562 as a measure of non-specific killing. High non-specific cytolytic activity developed by day 4-7 of culture and peaked at about day 10: two examples are shown in Fig. 3a. The results suggested that the cells killing K-562 were not simply surviving NK cells.

An increase in the responder to stimulator ratio did not affect the relative proportions of non-specific killer cells and cytolytic T cells which were generated (Fig. 3 a & b), but slightly altered the kinetics of the appearance of these cells. The addition of CM which was free of PHA but contained TCGF and interferon from the beginning of culture resulted in increased cell proliferation, but did not increase the levels of cytolytic activity (Fig. 3c).

Precursors of non-specific killer cells in blood

The cells generated in B lymphoblast stimulated cultures had a T cell phenotype, and few if any expressed NK cell markers. Despite this, it was assumed throughout this study that non-specific killer cells killed K-562 but not B lymphoblasts and, conversely, that after induction specific CTC killed B lymphoblasts but not K-562; thus, the killing of K-562 alone would provide a measure of

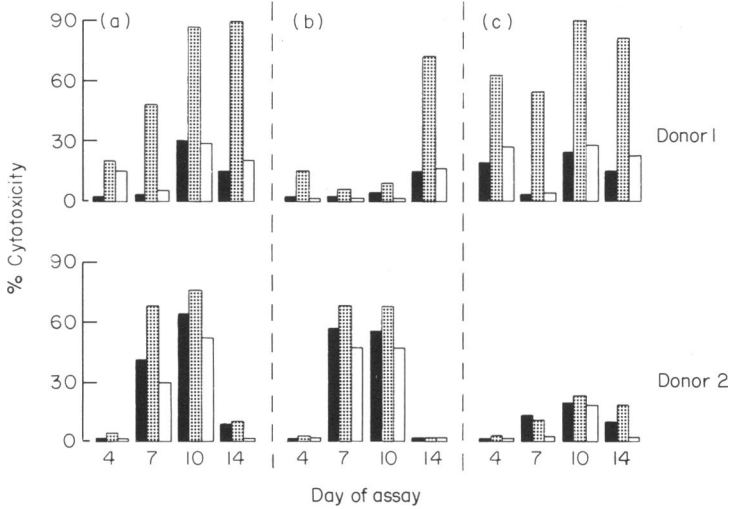


Fig. 3. Kinetics of the appearance of killer cells in *in vitro* culture. Blood cells were mixed with autologous irradiated B lymphoblasts at responder to stimulator ratios of 10:1 (a) or 40:1 (b) or at 10:1 with added CM on days 1, 4, 6, 9 and 12 (c). The results for two donors are shown after 4, 7, 10 and 14 days of *in vitro* co-culture. The *in vitro* generated killer cells were tested for their ability to kill autologous BL from the stimulating clone (■); K-562 (▨); and allogeneic BL (□) in a ⁵¹Cr release assay at a 10:1 effector:target ratio. The assays were performed in triplicate and the s.e. was always less than 2%.

non-specific cytolytic activity. This premise has been demonstrated indirectly by cold target inhibition studies by others but was formally shown by the following experiments. Preliminary studies at limit dilution indicated that the frequency among blood mononuclear cells of specific and non-specific killer cells generated in B lymphoblast stimulated cultures was less than 1 cell per 100. Therefore cultures were set up in microtitre plates with each well containing 5×10^4 irradiated autologous mononuclear cells as feeders, 1×10^4 irradiated autologous B lymphoblasts and 10 freshly isolated blood mononuclear cells. The medium was supplemented with 10% CM at the beginning of culture and every 3rd day, and the plates were incubated for 10 days. After this time the contents of each well were divided into two equal parts and distributed into different plates; the split clones thus obtained were tested against B lymphoblasts and K-562 cells in a ⁵¹Cr release assay, and the results of one such assay are shown in Fig. 4. Of 60 clones tested, 14 killed B lymphoblasts and

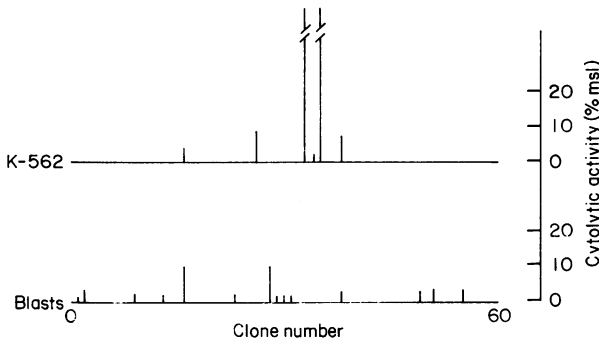


Fig. 4. The precursors of cells which kill K-562 are different from those which generate CTC. Cells were grown in mixed culture with autologous BL and CM and the resulting cells split into equal portions and tested for killing of BL and K-562 in a ⁵¹Cr release assay (msl = mean specific lysis). With only two exceptions, the clones which killed BL did not kill K-562 targets.

six killed K-562 and only two killed both targets; similar results were obtained when the assay was repeated on three further occasions.

DISCUSSION

Lymphocytes infiltrating human melanomas could be grown *in vitro* in liquid culture supplemented with TCGF. In six of seven attempts populations of lymphoblasts were grown directly from lymphocytes infiltrating the tumour and at the expense of melanoma cells which were destroyed in the process. These cultured lymphoblasts remained highly cytolytic towards fresh or cultured autologous tumour cells but, with time in culture, developed non-specific killer activity for various allogeneic tumour and other cell types, but not for autologous B lymphocytes.

Care was taken to exclude the possibility that lymphocytes grown from melanoma tissue were not the result of blood contamination; notably, during the first week of culture, the tumour derived cells displayed greater specific cytolysis for autologous tumour cells than did concurrently cultured blood mononuclear cells from the same patient. However, after 2 weeks of culture, the cytolytic properties of the tumour derived and blood derived killer cells were equivalent. The functional activity of the tumour derived lymphocytes cultured under the influence of TCGF was similar to that described in previous reports (Alvarez *et al.*, 1978) of human lymphocytes obtained from peripheral blood and maintained *in vitro* with TCGF i.e., non-specific cytolytic activity not restricted by histocompatibility antigens. This characteristic, together with the T cell phenotype of the cultured lymphoblasts, would identify these cells as activated lymphocyte killer (ALK) cells (Masucci, Klein & Argov, 1980). Hence further studies were performed to examine the *in vitro* conditions influencing the generation of ALK cells from cells in peripheral blood.

The nature and origin of ALK cells is controversial. The suggested origins include NK cells (Strassman, Bach & Zarling, 1983), T cells (Shortman *et al.*, 1983) or a distinct cell lineage (Grimm *et al.*, 1983). Vose & Bonnard (1982) and Vánky *et al.* (1982) demonstrated that blood lymphocytes activated by co-culture with autologous tumour cells can lead to the generation of specific CTC, but not of ALK cells, whereas activation by mixed lymphocyte culture or simply by growth in TCGF leads to generation of ALK cells (Seeley & Golub, 1978; Vánky *et al.*, 1982; Grimm *et al.*, 1983). In the present studies, peripheral blood cells were co-cultured with B lymphoblasts, and the kinetics of the appearance of ALK cells and of specific CTC, as measured respectively by the killing of K-562 and stimulating B lymphoblasts, were similar.

In agreement with other studies (Pawelec *et al.*, 1982; Grimm *et al.*, 1983), the surface markers of ALK cells after co-culture were found to be predominantly those of T cells and there was little expression, if any, of Leu 7 which is a marker which appears to encompass all fresh NK cells (Abo, Cooper & Balch, 1982). Clone splitting experiments clearly demonstrated that most of the killer cells which killed autologous B lymphoblasts failed to kill K-562, indicating that the two cytolytic cell types arose from different precursors. Further studies are required to ascertain whether ALK cells are derived from cytolytic T cells which differentiate in tissue culture in the absence of repeated antigenic stimulation and in the presence of high levels of TCGF, as suggested by Shortman *et al.* (1983) and Brooks (1983), or whether ALK cells represent the outgrowth of a previously unrecognized cell type (Grimm *et al.*, 1983).

The present work indicates that cytolytic lymphocytes with the characteristics of ALK cells can be cultured from within tumour tissue and that such cells can be generated under particular culture conditions from mononuclear cells in peripheral blood. These findings prompt further analysis of the properties of ALK cells with the expectation of better understanding of immunological mechanisms operative in the control of tumour cell growth *in vivo*.

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