Cancer Immunol Immunother (1983) 15:227–236

Cancer Immunology Immunotherapy

Tumour-reactive lymphocytes stimulated in mixed lymphocyte and tumour culture

Clonal analysis of effector cells in cytotoxic and proliferative assays

B. M. Vose and W. White

Department of Immunology, Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester, M20 9BX, England

Summary. *Lymphocytes from cancer patients were stimulated in mixed culture with autologous tumour (MLTC) or pooled allogeneic lymphocytes (MLC). Both protocols induced increased uptake of ^{3}H-thymidine at 5 days and the appearance of lymphoblasts. Blasts were isolated on discontinuous Percoll gradients and either expanded as bulk cultures or cloned directly under limiting dilution conditions in the presence of conditioned medium containing IL-2. Results with MLTC-blast-CTC have been reported elsewhere. MLC-activated cultures lysed autologous tumour but not autologous lymphoblasts. Lysis of some allogeneic tumours, lymphoblasts from members of the inducing pool, and K562 was also apparent. MLC activated cultures did not undergo restimulation in response to autologous tumour or lymphocytes but were restimulated by leukocytes from pool members.*

MLTC clones showed autologous tumour-specific cytotoxic activity or cross-reactive proliferative responses with tumours of the same site and histology. The majority of MLC clones cytotoxic for autologous tumour were also specific and did not lyse allogeneic tumour, K562, or lymphoblasts from the inducing pool. Two clones lysed autologous tumour and pool members. None of the clones tested proliferated in response to autologous tumour following MLC activation but some were responsive to pool members and one clone was restimulated by autologous monocytes. No association was found between clone phenotype and function. The implication of these data is that the effector cells with activity against autologous tumour induced in MLC arose largely by transstimulation of in vivo-activated tumour reactive lymphocytes by IL-2 release rather than expansion of NK-like effectors or sharing of antigenic specificities between tumour and allogeneic lymphocytes. Since MLC activation of cancer patients lymphocytes does not induce proliferative responses to autologous tumour it is unlikely to be a useful procedure in preparing cells for immunotherapy protocols.

Reprint requests should be addressed to B. M. Vose at his present address: Department of Immunology, Imperial Chemical Industries, Pharmaceuticals Division, Mereside, Alderley Park, Macclesfield, England

Abbreviations used in this paper: PBL, peripheral blood lymphocytes; TIL, tumour infiltrating lymphocytes; MLTC, mixed lymphocyte tumour culture; IL-2, interleukin-2; MLC, mixed lymphocyte culture; LSM, lymphocyte separation medium; BSS, balanced salt solution; HuSe, human serum; PBS, phosphate-buffered saline; CTC, cultured T cells; PHA, phytohaemagglutinin; CM, cultured medium; NK, natural killer; FcR, receptor for the Fc portion of IgG

Introduction

Some (20%–30%) patients with cancer were shown to have lymphocytes in the blood (PBL) and tumour-draining lymph nodes and within the tumour (TIL) which had specific cytotoxic activity against freshly isolated autologous tumour cells in short-term ^{51}Cr-release assays [18, 21, 30, 33]. Co-cultivation of lymphocytes with autologous tumour (mixed lymphocyte tumour culture, MLTC) induced (i) the appearance of lymphoblasts; (ii) DNA synthesis measurable by uptake of ^{3}H-thymidine [16, 17]; and (iii) the induction or augmentation of specific anti-tumour cytotoxicity [16–18, 31, 32]. These reactions were apparent in 60%–75% of patients. Taken together these data implied that autorecognitive tumour-reactive T cells were present in the patients: an observation which raises the possibility of their use in specific immunotherapy of human neoplasia [27]. We and others have recently shown that following activation in MLTC it was possible to expand cytotoxic T lymphocytes by culture in the presence of interleukin-2 (IL-2) and that cultured T cells retained their specificity for autologous tumour cells [15, 19, 22, 24]. In addition, it was possible to demonstrate that cultured T cells could be restimulated by autologous tumour cells in primed lymphocyte tests. Using IL-2 in limiting dilution assays it was also possible to enumerate precursors of both proliferative and cytotoxic T lymphocytes responding in MLTC. Approximately $^1/_{600}$ to $^1/_{1,000}$ PBL and $^1/_{200}$ to $^1/_{600}$ TIL proliferated in the presence of tumour, and of those one-half to one-quarter were cytotoxic for autologous tumour [22].

This enrichment of anti-tumour effectors at the tumour site considerably strengthens the view that in at least some individuals the tumour is indeed immunogenic and that homing or selective trapping of reactive lymphocytes can occur.

However, the induction of cytotoxic activity against freshly isolated autologous tumour is not restricted to MLTC-stimulated cultures. Sensitisation of patients' lymphocytes in mixed lymphocyte culture (MLC) [5, 14, 15, 35], by lectins [11] or by lectin-free IL-2 preparations without other stimulation [7, 9, 29] have been shown to induce autologous tumour cytotoxicity. Lysis of third-party targets was also found in these studies, so that the exact significance and nature of the cytotoxic effector remained unclear.

In the present study, we compared activation of patients' lymphocytes in MLC and MLTC to determine whether both proliferative and cytotoxic activities against autologous tumour were induced. Cloned cytotoxic and proliferative lines have been derived from MLC and MLTC to facilitate this

comparison. By use of these clones it has been possible to show that cytotoxic activity in MLC-activated populations was attributable largely to expansion of specific in vivo-activated T-cell populations by IL-2 release rather than expansion of NK-like effectors [7, 12], since tumour cell lysis in the absence of killing of K562 was found. The possibility that tumour and stimulators might share antigens [1, 2] appeared unlikely since clones showed specific reactivity with tumour and most frequently did not lyse allogeneic lymphoblasts.

Materials and methods

Lymphocytes. Lymphocytes from patients with cancer of the breast or colon were isolated from heparinised venous blood samples (30–40 ml) by centrifugation (900 *g*, 15 min, room temperature) of 10-ml aliquots on 10-ml gradients of lymphocyte separation medium (LSM Flow Laboratories, Irvine, Scotland). Mononuclear cells were harvested from the interface, washed twice in Hank's balanced salt solution (BSS) and resuspended in 20 ml RPMI 1640 supplemented with 10% heat-inactivated (56° C, 30 min) autologous plasma (HuSe), ampicillin (100 μg/ml), streptomycin (100 μg/ml), flagyl (10 μg/ml) and amphoteracin B (2.5 μg/ml). Adherent monocytes were depleted by incubation (30 min, 37° C) of cells in Costar 75-cm^2 culture flasks and non-adherent cells were enriched for T lymphocytes by passage through nylon wool columns [8]. Monocytes were isolated from the flasks by treatment with 0.2% EDTA in PBS containing 5% fetal calf serum for 10 min on ice.

Tumour cells. Tumour cell suspensions were prepared from tissue within 2 h of removal as previously described [23]. Tissue was finely minced and placed in an enzyme mixture consisting of collagenase (2 mg/ml), hyaluronidase (0.1 mg/ml), and DNAse (0.01 mg/ml) in Hank's BSS. All enzymes were obtained from Sigma Chemical Co., St Louis, Missouri, USA. The mixture was stirred slowly for 3 h, cells harvested, fresh enzyme mixture added, and dispersion continued until completed (overnight). Cells were washed twice in Hank's BSS, layered onto a 10-ml gradient of LSM (density 1.077), and centrifuged (900 *g*, 10 min). Cell debris, dead cells, and erythrocytes passed through LSM to yield a heterogenous but largely viable population at the interface. Cells were washed twice in Hank's BSS, resuspended in 20–40 ml RPMI + 10% HuSe, and incubated for 4 h at 37° C in 75-cm^2 Costar culture flasks to allow regeneration of cell surface components. Adherent cells consisted primarily of macrophages (non-specific esters positive, FcR-positive, phagocytic) [20], although occasionally small numbers of tumour cells did adhere under these conditions. Non-adherent cells were applied to a two-stage gradient comprising 6 ml LSM overlayed with a 3 : 4 dilution of LSM in PBS (density 1.055). After centrifugation (900 *g*, 10 min, room temperature) a lymphocyte-enriched population was taken from the LSM interface and a tumour cell-enriched layer isolated on the upper interface. Tumour-infiltrating lymphocytes were purified further by passage through nylon wool columns to yield a population of > 95% lymphocytes more than 85% of which were OKT3-positive. The tumour cell-enriched layer (in 2 ml RPMI) was layered onto HuSE (8 ml) and allowed to stand for 2 h at 22° C. Tumour cells had a high sedimentation rate (1 cm/h) and could thus be separated from contaminating lymphocytes. Tumour cells were used as stimulators in mixed lymphocyte-tumour cultures (MLTC) or cryopreserved (3–5 × 10^6 cells in 2 ml) over liquid nitrogen by controlled rate freezing (1° C/min) in the presence of 10% dimethylsulphoxide and 25% autologous, heat-inactivated plasma. Cryopreserved cells were recovered by rapid thawing in a 37° C water bath and slow dilution to

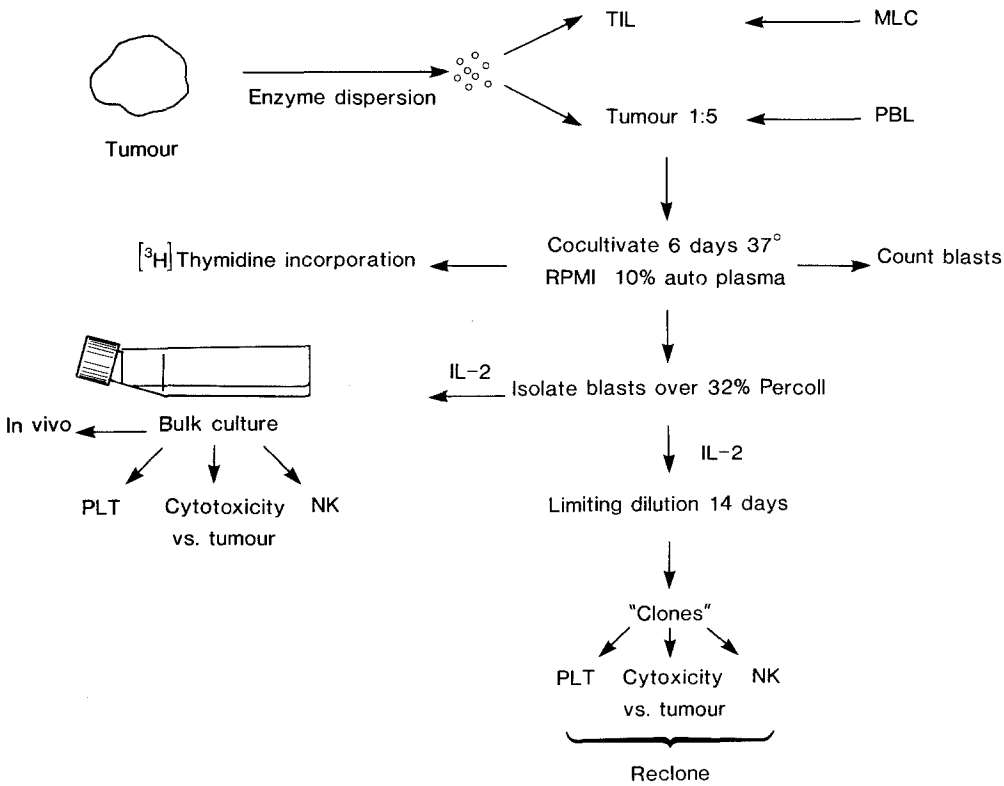

Fig. 1. Experimental design

15 ml in RPMI + 10% HuSe over a 10-min period. If necessary, non-viable cells were removed from recovered cells by centrifugation on LSM gradients as described above.

Experimental design. The experimental design is illustrated in Fig. 1. Patients' blood lymphocytes or TIL were stimulated by autologous tumour cells (MLTC) or in mixed lymphocyte culture (MLC); lymphoblasts were separated and placed in bulk culture in flasks or cloned. Cultures and clones were tested for specific cytotoxicity against autologous tumour, NK-like activity against the K562 cell line, restimulation in primed lymphocyte tests, and phenotyped. To date, it has not been possible to reclone populations.

MLTC/MLC. Patients' blood- or tumour-infiltrating lymphocytes were dispensed into Titertek 96-well round-bottomed plates (1×10^5 cells/well) in RPMI 1640 + 10% HuSe. Stimulators were irradiated (3,000 R) and added to wells, after which the fluid volume was adjusted to 0.2 ml. Stimulators used were: autologous tumour (stimulator : responder ratio 1 : 5); autologous monocytes to control for autologous MLR (stimulator : responder ratio 1 : 5); and lymphocytes pooled from four healthy donors (BMV, KR, WW, HH) in equal numbers and used at a pool stimulator : responder ratio of 1 : 1). Plates were incubated for 5 days at 37° C and pulsed for the final 18 h of culture with 1 μCi/well ^{3}H-thymidine (specific activity 2.5 μCi/mMol, Radiochemical Centre, Amersham, Bucks.). Cells were harvested onto fibre discs with a Scatron multiple culture harvester and radioactivity measured in a β-counter with a toluene-based scintillant. MLTC was considered positive when the stimulation index (uptake ^{3}H-thymidine in test/uptake ^{3}H-thymidine in controls) was greater than 3. Standard deviation on the triplicate samples was less than 10% of the mean.

Bulk MLTC/MLC. Responder cells (1×10^6/ml in RPMI 1640 + 10% HuSe) were mixed with stimulators (2×10^5/ml tumour cells or 1×10^6/ml pooled allogeneic leukocytes) and incubated for 6 days at 37° C in Costar 25-cm^2 culture flasks standing upright. After incubation cells were washed twice in RPMI 1640, blasts counted and isolated on discontinuous Percoll gradients. Gradients consisted of 5 ml (40%) Percoll in RPMI 1640 + 10% HuSe 1.5 ml (35%), 2.5 ml (31.5%), and 2.5 ml (26%). Cells in 1.5 ml were layered onto this and centrifuged (550 *g*, 30 min, room temperature). Blast cells were taken from the 31.5% interface and used to initiate bulk cultures or cloned by limiting dilution.

Conditioned media (CM) containing interleukin-2. Human spleens removed for access in patients undergoing surgery for gastric carcinoma were chopped and tissue pressed through stainless steel grids. Mononuclear cells were separated on LSM gradients and stimulated with PHA (1 μg/ml) at a cell concentration of 2×10^6/ml in RPMI 1640 + 1% HuSe. Supernate was collected after 24 h incubation at 37° C and precipitated at 50% saturation by slow addition of 312 g $(NH_4)_2SO_4$/l. After stirring for 2 h at room temperature, material was centrifuged (10,000 *g*, 30 min) and precipitate which contained PHA was discarded. The supernate was precipitated at 75% saturation by addition of a further 156 g/l $(NH_4)_2SO_4$. Precipitate was collected, resuspended in double-distilled water, and dialysed against two changes of water and one of RPMI 1640. Volume was adjusted to 20% of the starting volume. Conditioned media were tested for IL-2 activity by their capacity to induce proliferation and maintain long-term growth of an IL-2-dependent human T-cell line. In all cultures CM were used at the lowest concentration giving maximal stimulation of the line (usually 10% v/v).

Bulk cultures. Isolated blast cells (3×10^5/ml) were expanded in RPMI 1640 + 10% HuSe supplemented with 10% CM. Cultures were counted every 3–4 days and adjusted to 3×10^5/ml by addition of RPMI 1640 + 10% HuSe and CM. Prior to use in cytotoxicity or proliferation assays cells were washed free of CM and incubated overnight to avoid non-specific lytic activity [12] and reduce background counts in PLT.

Cloning. Blast cells were dispensed into Titertek 96-well round-bottomed culture plates at different dilutions (between 200 and 0.5 cells/well together with 10% CM and 2.5×10^4 pooled irradiated (3000R) MLC stimulator cells. After 14 days' incubation (37° C, 5% CO_2, humidified atmosphere) plates were examined and wells with obvious growth marked. A plot was made of log percentage wells negative against number of cells plated, and the limiting frequency was determined as the number of cells plated corresponding to 37% wells negative. 'Clones' were taken where 10% or less of wells were positive. Proliferative frequency was confirmed by testing parallel plates for uptake of ^{3}H-thymidine [22]. Cells from postive wells were placed in 1-ml culture in RPMI 1640 + 10% HuSe + 10% CM with 2.5×10^5 pooled MLC stimulators as feeders. Cultures were maintained in 1 ml by addition of fresh medium (0.1 ml CM) and 2.5×10^5 feeders at weekly intervals. Cells grew at a concentration of approximately 5×10^5 to 1×10^6/ml. It was possible to maintain cells for up to 12 weeks in this way, but cells then lost responsiveness to CM and died in spite of the continuous presence of stimulator cells. It was for this reason that recloning of positive wells was not possible in this series.

Cytotoxicity. Cytotoxic activity of CTC was assessed in short-term (4 h) ^{51}Cr-release assays against freshly isolated or cryopreserved tumour cells as previously described [21]. Tests were also performed against PHA-induced lymphoblasts (5-day stimulation, 1 μg/ml PHA, washed 1 day prior to assay) of the pool stimulators. Autologous monocyte preparations were used as targets in some experiments. Cytotoxicity was considered positive when it was dose-dependent and exceeded spontaneous release by at least three standard deviations.

Primed lymphocyte tests (PLT). Cultured T cells (5×10^3 to 1×10^5/well) in 96-well round-bottomed plates were stimulated with 5×10^4 irradiated (3000R) stimulator cells for a 48-h period and pulsed with ^{3}H-thymidine over the last 6 h of culture.

Phenotype. Phenotype of clones was monitored with the monoclonal antibodies OKT3 (pan T reagent), OKT4 (helper/inducer), and OKT8 (suppressor/cytotoxic) obtained from Ortho Pharmaceutical Company, Rariton, NJ and visualised with FITC-labelled rabbit anti-mouse immunoglobulin (DAKO Mercia Brocades Limited, Weybridge, Surrey, England).

Results

Stimulation of patients lymphocytes in MLTC and MLC

Results of large series of MLTC from this laboratory have been reported previously [24, 28]. Table 1 presents recent data from experiments with nine breast and two colon cancers with different stimulator protocols. Nylon wool-passed blood lymphocytes or TIL were co-cultivated with autologous tumour, autologous monocytes, and pooled allogeneic lymphocytes. Unstimulated cultures consisted of lymphocytes cultured in the presence of irradiated nylon wool passed lymphocytes to control for the effect of differing cell density. Six breast cancer patients (nos. 187, 180, 179, 158, 154, 148) and one colon cancer patient (90) showed significantly increased uptake of ^{3}H-thymidine following exposure to autologous tumour cells in at least one lymphocyte preparation. Levels of stimulation varied markedly so that positive stimulation indices (s.i. defined in *Materials and Methods*) ranged from 4.6 to 41. The percentage of surviving cells with lymphoblastic morphology was closely correlated with stimulation index and ranged from 4% in patient 179 (s.i. 4.6) to 25% in patient 148 (s.i. 46). Stimulation by autologous tumour

Table 1. Stimulation of patient's lymphocytes (Ly) from blood (PBL) or tumour (TIL) with autologous tumour cells, monocytes, or pooled allogeneic mononuclear cells

Responder		Uptake of ^{3}H-thymidine[a] in the presence of			
		Ly Alone	Ly + Tu	Ly + pool	Ly + monocytes
Breast	PBL 187	292	7,296 (25)	66,671	1,889 (6.4)
	TIL 187	130	824 (6)	9,639	214 (1.6)
	PBL 182	1,404	1,874 (1.3)	4,094	–
	PBL 180	902	6,673 (7.4)	13,592	–
	PBL 179	283	1,303 (4.6)	–	–
	PBL 158	202	298 (1.5)	–	–
	TIL 158	115	4,770 (41)	–	–
	PBL 154	562	460 (0.8)	–	–
	TIL 154	1,127	6,203 (5.5)	–	–
	PBL 150	960	1,193 (1.2)	–	–
	PBL 148	550	10,090 (18)	–	487 (0.9)
	PBL 185	4,623	2,501	34,416	1,785
Colon	PBL 90	3,444	37,758 (11)	58,529	
	PBL 86	2,098	1,811 (0.9)	22,515	

[a] cpm
Numbers in parenthesis indicate stimulation indices.
Breast cancer patients 148, 158, 179, and 182 presented with secondary disease as chest wall recurrence or skin nodules
Uptake of ^{3}H-thymidine by stimulator cells alone was less than 300 cpm and was subtracted from the values shown

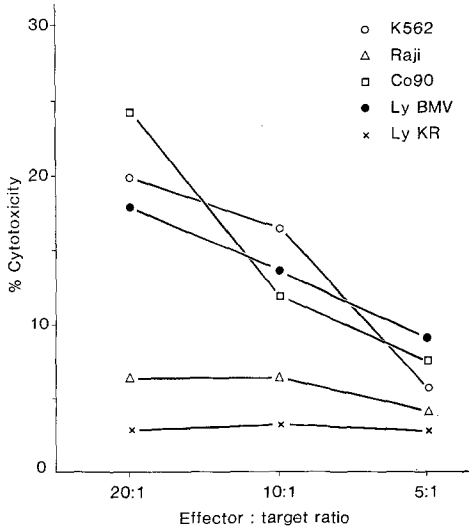

Fig. 2. Cytotoxicity of MLC-blast-CTC against various targets including autologous tumour (Co90) and lymphoblasts from two of the sensitising pool. NK-sensitive (K562) and -resistant (Raji) cell lines were included

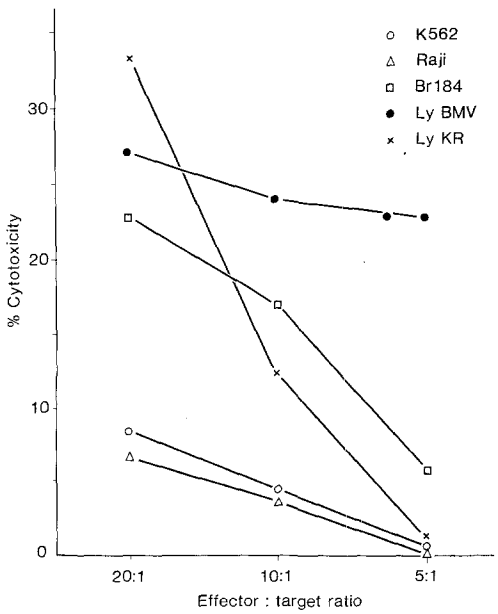

Fig. 3. Cytotoxicity of MLC-blast-CTC against autologous breast tumour (Br 184) lymphoblasts of the sensitising pool (Ly BMV and Ly KR) and cell line targets

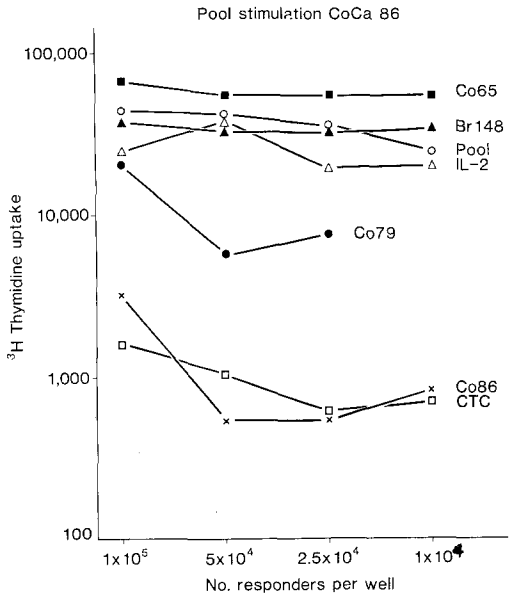

Fig. 4. Primed lymphocyte test of pool-sensitised MLC-blast-CTC against autologous tumour (Co86) allogeneic fresh tumour cells (Co65, Co79, Br148) and the sensitising pool. IL-2 was included as a positive control. Uptake of ^{3}H-thymidine was assessed by a 6-h pulse after 48 h stimulation

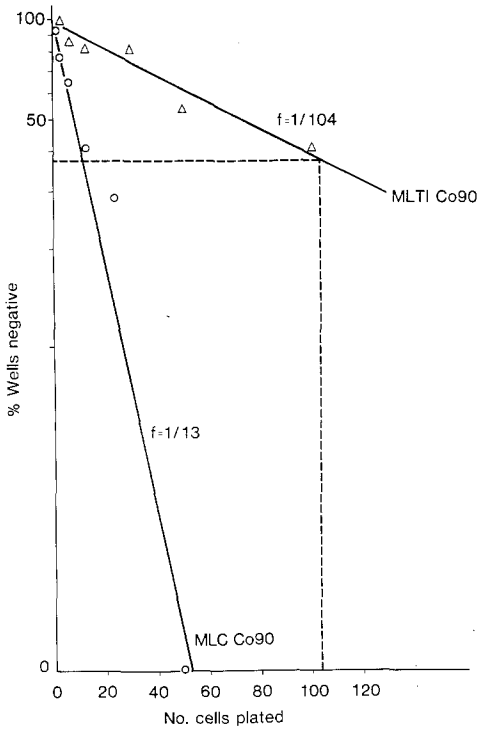

Fig. 6. Limiting dilution analysis of the frequency of clonogenic cells responding to IL-2 in isolated blast fractions following MLC or MLTC stimulation. Cells were plated in four 96-well round-bottomed plates with pooled irradiated allogeneic feeders (2.5 × 10^4/well) and conditioned medium. Visible cell growth was scored at day 14

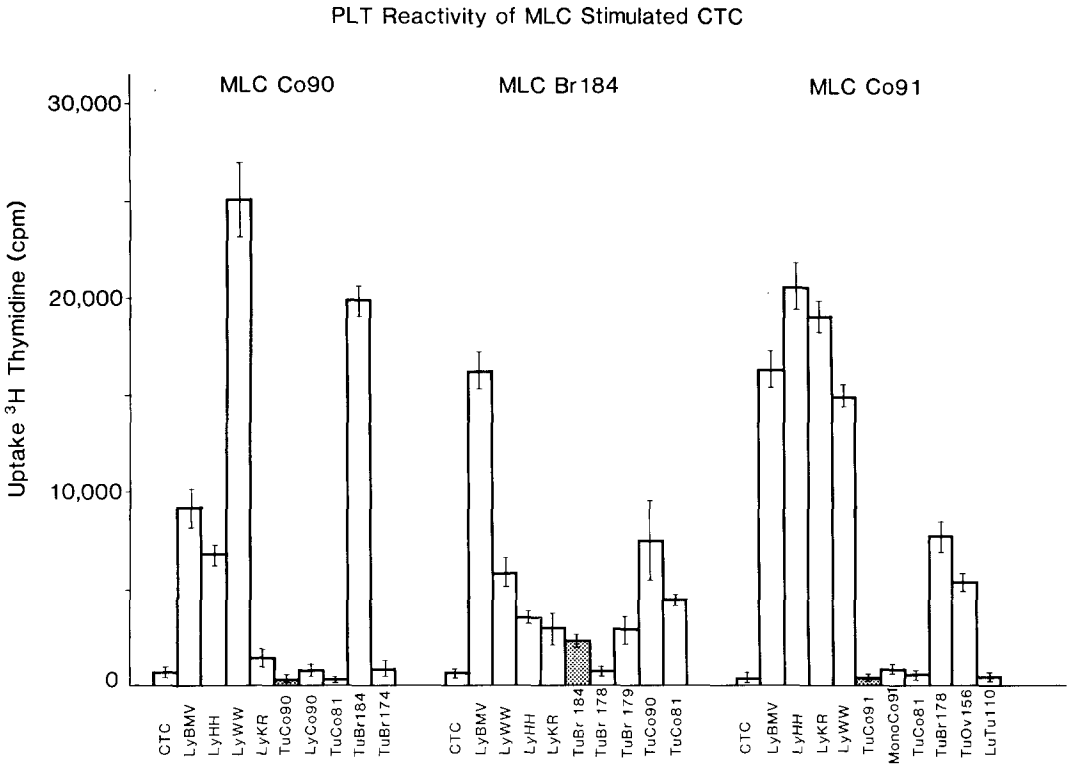

Fig. 5. PLT reactivity of three MLC-blast-CTC against individual pool sensitisers (Ly BMV, Ly HH, Ly WW, Ly KR), autologous tumour (Tu), lymphocytes (Ly), monocytes (Mono) and allogeneic tumours. Response to autologous tumour *shaded*. *Bars* indicate standard deviations of triplicate determinations

of lymphocytes from the different sites was variable and was positive in TIL in each of the three breast cancer patients tested (187, 158, 154), even in cases (158 and 154) in whom PBL showed no significant increase of ^{3}H-thymidine uptake. In this series of breast cancers, material was obtained from four patients with secondary disease (chest wall recurrence or skin nodules). Stimulation of PBL was recorded in two of these (148 and 179). Lymphocytes from the six patients tested responded to pooled allogeneic feeders, with thymidine uptake often exceeding peak stimulation with phytohaemagglutinin (data not shown). Up to 60% of surviving cells following MLC were lymphoblasts. Autologous MLR with monocytes as stimulators was recorded in patient 187's PBL. In keeping with our previous studies stimulation by tumour invariably exceeded that induced by autologous monocytes [24]. MLTC/MLC microcultures were completed on day 5. In those cases showing positive MLTC in ^{3}H-thymidine tests, parallel bulk cultures were processed on day 6. Following isolation of blasts on Percoll gradients MLC-blast-CTC and MLTC-blast-CTC were initiated.

MLC/MLTC-Blast-CTC

The reactivity of MLTC-blast-CTC has been described previously [24]. In previous studies it was shown that these cultures had the capacity to lyse autologous but not allogeneic tumour and most frequently did not lyse autologous monocytes or lymphoblasts. Variable activity against the K562 cell line was demonstrable. In PLT, cultures underwent stimulation upon reexposure to autologous tumour and allogeneic tumour of the same organ and histology, but not to other tumours, monocytes or lymphocytes.

Cultured T cells from MLC-stimulated lymphocytes lysed autologous tumour cells. Data from two representative examples of the six cultures tested are presented in Figs. 2 and 3. Lymphoblasts from members of the sensitising pool, allogeneic tumours (not shown), and the K562 cell line were also killed, i.e., there was widespread lytic activity. However, autologous monocytes and lymphoblasts were not killed.

Primed lymphocyte tests with MLC-blast-CTC were also performed (Fig. 4). Cultures underwent restimulation with IL-2 (positive control), with the inducing stimulator pool, and most frequently with each member of it. Some allogeneic tumours induced increased ^{3}H-thymidine uptake but this was not seen with autologous tumour, lymphocytes, and monocytes. Results with three further examples of the six cases tested are presented in Fig. 5. Patterns of reactivity were relatively constant although occasionally one member of the pool did not induce DNA synthesis (e.g., MLC Co90 with LyKR, Fig. 5). In only case (MLC Br 184, Fig. 5) was there evidence of restimulation with autologous tumour (s.i. 3.5).

Cloning of MLTC/MLC

To investigate the reactions described above, we have cloned effector cells. Blast preparations from MLTC and MLC were plated under limiting dilution conditions in Titertek 96-well round-bottomed plates in the presence of pooled allogeneic blood mononuclear cells as feeders and conditioned medium containing IL-2. After culture for 14 days growth was apparent, so that enumeration of positive wells was possible. From this scoring a plot of log percentage wells negative against number of cells plated could be made and the responder frequency thereby derived as the concentration of cells corresponding to 37% of wells negative (Fig. 6). Regression lines intercepted the y-axis at approximately 100%.

In five MLTC-positive cases where clones were successfully initiated cloning efficiency of MLC blasts plated was $^1/_{13}$ to $^1/_{24}$ and $^1/_{27}$ to $^1/_{104}$ in MLTC blasts. Once positive wells had been identified and transferred to 24-well Costar plates the majority grew to give large cell numbers within 1 week. Cells

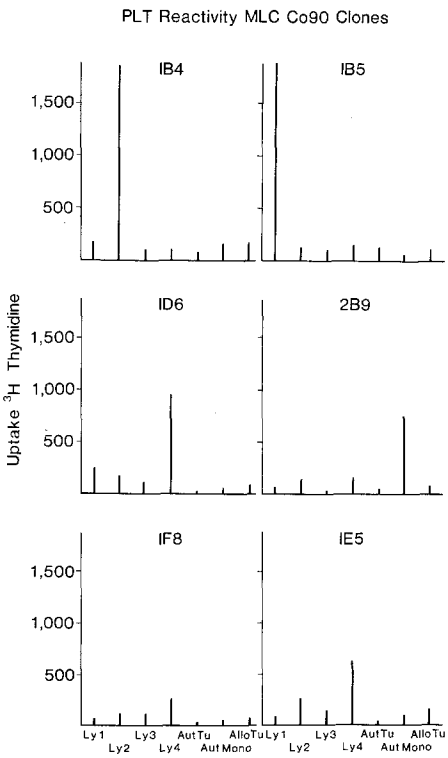

Fig. 7. Primed lymphocyte test of reactivity of six MLC clones (5×10^3 cells plated) against sensitising pool members (Ly 1, 2, 3, 4), autologous (Aut Tu) and allogeneic (Allo Tu) tumour, and autologous monocytes Aut Mono). Stimulators were added to give 5×10^4 cells/well

Table 2. Reactivity of clones from MLTC-stimulated cultures against various cells in primed lymphocyte tests

Clone	(^{3}H)-Thymidine uptake with					
	Autologous tumour	Allogeneic tumour	Autologous monocyte	Lymphocyte 1	Lymphocyte 2	Lymphocyte 3
12	2,386*	2,147*	380	683	580	672
13	4,187*	1,365*	633	875	619	713
20	2,482*	712	775	436	450	–
21	450	877	572	2,814*	1,229	775
17/3	5,710*	4,700*	217	481	504	403

* Significant stimulation – cpm

Allogeneic tumour was matched with autologous for site and histology. Clones 12, 13, 20, colon carcinoma; clone 17/3 squamous carcinoma of lung. Clones 12, 13, 20 did not respond to breast adenocarcinoma cells. Clone 17/3 did not react with adenocarcinoma of lung or breast, AML blasts, or ovarian ascites, but did react with three squamous carcinomas of lung

were used in cytotoxicity and proliferation assays so that culture volume was maintained at 1–2 ml (2×10^5 to 1×10^6 cells) by addition of fresh pooled feeders at weekly and IL-2 at twice-weekly intervals until response to IL-2 was lost. With over 100 clones so far examined none has survived in culture beyond 12 weeks.

In proliferation assays of MLC-activated clones, cultures were indentified which underwent secondary stimulation in response to a single member of the inducing pool, e.g., clones IB4, IB5, ID6 (Fig. 7). Many clones (31 of 46) showed no proliferative activity (e.g., clone IF8, Fig. 7). Some clones reacted to more than one stimulator (IE5 reacting weakly but significantly with lymphocytes 2 and 4). Since all cultures were derived from wells seeded with several cells it is not clear whether this is a true cross-reactivity or simply a result of growth of more than one cell in the well. A single clone was reproducibly restimulated by autologous monocytes (2B9, Fig. 7). None of the clones responded to autologous tumour: a finding in accord with the results with bulk MLC-blast-CTC. Some MLTC-derived clones were stimulated by autologous tumour (Table 2) and by allogeneic tumour of the same site (clones 12, 13, and 17/3). They did not generally respond to allogeneic lymphocytes, except for clone 21, which responded to lymphoblasts from a pool member used in the feeding protocol.

In cytotoxicity assays with clones from MLTC-stimulated cells as effectors, reactivity was regularly found against cells of the autologous tumour in the absence of similar reactivity against allogeneic tumours or the K562 cell line (Table 3). Of 11 clones tested, six showed significant reactivity against autologous tumour with cytotoxicity between 14.8% and 26.5% at an effector : target ratio of 5 : 1. One clone (11) showed significant reactivity against autologous and allogeneic tumour of the same type and one (13) showed low, but significant cytotoxicity against LyBMV blasts, which were one of the pooled allogeneic feeders used in the maintenance of clones. We are initiating further clones to examine this reaction more fully. None of the MLTC clones had both proliferative and cytotoxic activity. Clones derived from pooled MLC-stimulated cultures showed a more varied pattern of cytotoxic reactivity (Table 4). Six clones lysed autologous tumour but not allogeneic tumour, K562, or cells of the stimulatory pool. Many other clones, only some of which are presented in Table 4, lysed single members of the stimulating pool. Two clones reproducibly lysed LyWW and LyKr blasts. Clone $Co90_{18}$ lysed allogeneic tumour and lymphocytes from a single pool member. Two clones (Co 91/22 and Co 90/21) showed both cytotoxic and proliferative activity against an allogeneic pool member. Upon repeat testing 2 weeks apart the patterns shown in Tables 3 and 4 were shown to be stable but further testing was frequently limited by lack of autologous tumour material. In this series no clones with reactivity against the K562 cell line were detected, although we and others have previously found several such clones after MLC stimulation [13, 25, 26, 34].

Phenotype

Over 95% of cells in all clones were reactive with OKT3 and formed rosettes with sheep erythrocytes They did not react with OKM1, Leu 7, or anti-human immunoglobulin. When tested for reactivity with OKT4 and OKT8 one clone, $MLTC90_{18}$ (autologous cytotoxic), reacted with neither antibody, and clone MLC 91/5 (autologous cytotoxic) reacted with both OKT4 and OKT8, over 90% of cells staining with each monoclonal. Other clones showed predominant reactivity with one antibody but there was no association between phenotype and function (Table 5). For example, OKT4-positive (23,90/17, 90/22) and OKT8-positive (91/12, 90/25) lines

Table 3. Cytotoxic activity of clones from MLTC-stimulated cultures against various targets

Clone	% Cytotoxicity[a] against						
	Autologous tumour	Allogeneic tumour	K562	Lymphoblasts			
				BMV	KR	WW	HH
3	18.9[c]	–[d]	0.2	–	–	–	–9.1
4	19.4[c]	–	–0.8	2.1	6.1	–2.1	1.2
11	15.6[c]	17.8[c]	–	–	–	–	–7.5
12[b]	7.2	5.7	1.7	–	–	–	–
13[b]	3.9	–	–0.9	10.6[c]	7.8	4.5	6.0
17	5.9	–	0.4	–	–	–	3.5
18	14.8[c]	5.6	–	–	–	–	–
19	26.5[c]	2.8	1.6	–	–	–	–
20[b]	9.4	–	1.8	–2.1	–1.3	–1.7	–4.8
21[b]	0	–	3.6	0.2	–	–4.0	–2.8
23	18.0[c]	–9.3	–	–	–	–	9.3

[a] Effector: target ratio 5:1
[b] PLT-positive clones
[c] Cytotoxicity was considered significant when ^{51}Cr release in test samples exceeded spontaneous ^{51}Cr release by more than three standard deviations
[d] Not tested

Table 4. Cytotoxic activity of clones from pool MLC-stimulated cultures against various targets

Clone	% Cytotoxicity[a] against						
	Autologous tumour	Allogeneic tumour	K562	Lymphoblasts			
				BMV	KR	WW	HH
90/ 4	12.3	2.2	–	–	–	–	–
91/ 8	– 2.5	– 8.7	–	–	–	–	–
90/10	3.3	–	–	–	–	–	–
90/15	– 4.2	–	–2.6	22.8[b]	6.3	– 8.7	– 7.9
90/16	– 1.4	–	–2.8	17.1[b]	1.9	14.9[b]	– 6.7
90/17	23.0	– 1.5	–1.5	1.2	–	–	3.5
90/18	– 6.3	53.5	–	–	–	–	–
90/21	2.6	–	–0.4	2.1	46.7[b]	– 1.5	–10.5
90/22	20.9[b]	– 8.2	0.5	–	–	–	5.9
90/23	20.7	– 1.4	1.0	–	–	–	– 6.0
91/ 2	21.4[b]	–	0.2	0.7	–	–	
91/ 3	– 4.6	–	0.4	1.2	–	–	
91/ 5	69.8[b]	7.4	1.3	–	–	–	3.5
91/ 6	13.9	–	–3.3	9.6	10.3[b]	20.8[b]	1.2
91/ 7	9.6	–	–4.9	0.2	8.2	0.8	6.5
91/ 8	– 1.7	–	2.0	4.9	– 0.6	2.1	6.2
91/ 9	– 2.7	–	–0.5	0.3	29.0[b]	17.7[b]	4.0
91/10	– 5.3	–	–5.4	1.1	– 0.4	0	0.2
91/12	20.4[b]	4.8	–5.2	0	4.1	2.5	1.0
91/13	17.8[b]	–	–2.2	0.7	4.4	3.8	13.9[b]
91/15	–11.3	–	–3.6	6.7	1.2	– 3.2	0
91/16	– 9.9	–	–5.2	4.0	7.2	18.2[b]	3.7

[a] Effector: target ratio 5:1
[b] Significant cytotoxicity (see footnote to Table 2)

Table 5. Phenotype of MLTC and MLC clones

Clone	Cytotoxic[a]	Proliferative[a]	% Cells stained with	
			OKT4	OKT8
MLTC				
12	−	+	87	1
21	−	+	0	94
23	+	−	97	0
90/18	+	−	2	4
MLC				
91/ 3	−	+	0	98
91/ 5	+	−	95	97
91/12	+	−	1	94
91/15	−	−	1	100
90/17	+	−	91	0
90/22	+	+	84	3
90/23	+	−	1	79

[a] Cytotoxic and proliferative activity against autologous tumour or lymphoblasts

showed cytotoxicity against autologous tumour or lymphoblasts.

Discussion

Lymphocytes with cytotoxic activity against autologous tumour cells could be cultured from MLTC- and MLC-activated populations in conditioned medium containing IL-2. Whereas MLTC-derived CTC were specific for autologous tumour [19, 24], MLC-blast-CTC lysed autologous tumour, some allogeneic tumours and lymphoblasts but not autologous lymphoblasts. NK-sensitive cell lines were also lysed to some extent by MLC- and less frequently MLTC-derived CTC. Similar data have been reported by several groups [5, 14, 19, 35]. At least three mechanisms have been proposed by which cytotoxic activity against autologous tumour could arise following MLC activation: (1) Expansion of NK-like cells and their activation by IL-2 or interferon [7, 12, 26]; (2) the presence on autologous tumour cells of 'inappropriate' histocompatibility antigens so that pool stimulation induces specific effectors with cross-reactivity between tumour and at least one pool member [1, 2, 35]; and (3) the expansion of lymphocytes which in vivo exposure to tumour had induced to express the IL-2 receptor by factor released during the MLC [15, 32]. In the present study we have attempted to distinguish effectors with different patterns of reactivity by cloning.

Clones were derived from blast cell preparations by seeding into wells in the presence of conditioned medium containing IL-2 and pooled allogeneic mononuclear cells as stimulators and feeders. Under these conditions growth of lymphocytes was regularly seen but the efficiency of expansion was not high, with between $^1/_{10}$ and $^1/_{25}$ cells from MLC and $^1/_{25}$ and $^1/_{100}$ from MLTC growing. Since all cultures came from wells containing multiple responders the assignation of clonality was on a statistical basis and no population was recloned. However, the majority of wells were monofunctional and had a clonal phenotype. Examination of the frequency of IL-2-responsive cells in limiting dilution assays showed that approximately $^1/_{5,000}$ PBL from cancer patients respond without stimulation [22]. This figure is close so that for healthy donors: $^1/_{4,500}$ to $^1/_{6,300}$ [26]. Following MLTC stimulation $^1/_{600}$ to $^1/_{1,500}$ cells can be expanded in IL-2 [22], and after blast isolation this rose to $^1/_{25}$ to $^1/_{100}$. Considerable enrichment of activated cells can thus be achieved with the methodology described here. Clones were expanded in 1-ml cultures and used as effectors in cytotoxicity assays.

MLTC-stimulated clones corresponded in reactivity to the MLTC-blast-CTC previously described [24]: they lysed autologous tumour but only rarely were allogeneic tumour or lymphoblasts killed (Table 3). Clones from MLC-stimulated cultures also lysed autologous tumour (8 of 21 clones). Interestingly, in this series none of the clones reacted with the K562 cell line, although in previous experiments K562 killer clones have been detected both from MLC-stimulated cultures [13, 31] and from the isolated large granular lymphocyte fraction [25]. The reason for our failure to detect such NK-like clones here is not clear, but may be attributable to careful isolation of blasts. These data suggest that in this induction protocol activation or expansion of NK-like effectors is not responsible for the autologous tumour lysis observed. Of the autologous reative clones six showed specific reactivity as far as tested with no significant reactivity against allogeneic tumour, K562, or lymphoblasts. Two clones (91/ and 91/13) reacted also with lymphoblasts from two and one member of the stimulating pool, respectively. Since these cultures have not been recloned it is difficult to draw firm conclusions from these observations. However, it appear from these data that induction of autologous tumour killing following MLC was the result of expansion of a tumour-specific lytic population, presumably by IL-2 released during stimulation. Precursors could have the IL-2 receptor induced by in vivo exposure to tumour. In addition, some inappropriate expression of shared specificities between tumour and allogeneic stimulator cells may be implied if it is considered that the cross-reactive cultures reacting with tumour and pool stimulators are indeed clonal. The latter possibility has been discussed in a recent paper [2], but recloning would be necessary to establish this. The cytotoxic activity of the majority of the clones corresponded in specificity with the patterns described by several groups where MLC-activated autologous tumour lysis was blocked in cold target inhibition assays by addition of tumour cells but not by lymphocytes of members of the stimulator pool [5, 14, 19].

In contrast to MLTC-blast-CTC [19, 24], MLC-blast-CTC did not show restimulation upon re-exposure to cells of the autologous tumour although they were restimulated by members of the stimulating pool and by some allogeneic tumours. Clones again showed reactivity corresponding so that of the bulk culture in that none of 46 clones from five patients proliferated upon re-exposure to autologous tumour. Proliferative clones generally responded to a single pool member and some allogeneic tumours. One clone was restimulated by autologous monocytes, presumably an indication of autologous MLR. The lack of proliferative activity in MLR was unexpected since good cytotoxic reactivity was recovered. This observation may have important consequences for the use of cultured T-cell clones or cultures in immunotherapeutic protocols. There is emerging evidence that in several models in which T lymphocytes were injected it was the proliferative rather than the cytotoxic subsets that induced tumour or graft rejection [3, 4, 6, 10]. Although technically easier to perform than autologous tumour stimulation, MLC stimulation does

not appear to induce appropriate reactivity should the same restrictions apply in human disease.

In this report we have attempted to delineate patterns of anti-tumour reactivity at the clonal level and succeeded in isolating clones with specific anti-tumour reactivity from both MLTC- and MLC-activated populations. Attempts to characterise clones further have been hampered by difficulties in maintaining long-term growth of cultures; all clones have lost responsiveness to IL-2 and died within 3 months of initiation. This represents a major limitation to in vivo use of CTC in immunotherapy. We are now attempting to resolve this problem with the use of different feeder/stimulator protocols and different sources of IL-2. It is envisaged that once these technical problems are overcome it should be possible to make rapid progress towards the characterisation and study of the fine specificity of human tumour-associated antigens.

Acknowledgements. This work was supported by a grant from the Cancer Research Campaign of Great Britain.

The authors are grateful to Mr. P. F. Schofield, Dr. N. Carr, Dr. A. Howell and Dr. M. Wilkinson for provision of tumour material. To Dr. M. Moore for encouragement and to colleagues in the laboratory for donation of blood.

References

1. Bach FH, Bach ML, Zarling JM (1978) Pool-priming: A means of generating T lymphocytes cytotoxic to tumour or virus-infected cells. Lancet I: 20
2. Bach FH, Leshem B, Kuperman OJ, Bunzendahl H, Alter BJ, Zarling JM (1982) Allosensitisation to obtain anti-tumour immunity. In: Fefer A, Goldstein A (eds) The potential role of T cells in cancer therapy. Raven Press, New York
3. Dallman MJ, Mason DW (1982) Role of thymus-derived and thymus-independent cells in murine skin allograft rejection. Transplantation 33: 221
4. Fernandez-Cruz E, Woda BA, Feldman JD (1980) Elimination of syngeneic sarcomas in rats by a subset of T lymphocytes. J Exp Med 152: 823
5. Fossati G, Baksari A, Taramelli D, Sensi ML, Pellegris G, Nava M, Parmiani G (1983) Lysis of autologous human melanoma cells by in vitro allosensitised peripheral blood lymphocytes. Cancer Immunol Immunother (in press)
6. Greenberg PD, Cheever MA, Fefer A (1981) Eradication of disseminated murine leukaemia by chemoimmunotherapy with cyclophosphamide and adaptively transferred immune syngeneic Lyt 1^+2^- lymphocytes. J Exp Med 154: 952
7. Grimm EA, Mazumder A, Zhang HZ, Rosenberg SA (1982) Lymphokine-activated killer cell phenomenon. Lysis of natural killer resistant fresh solid tumour cells by interleukin-2-activated autologous human peripheral blood lymphocytes. J Exp Med 155: 1823–1841
8. Julius MH, Simpson E, Herzenberg LA (1973) Rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur J Immunol 3: 645–649
9. Lotze MT, Grimm EA, Mazumder A, Strausser JL, Rosenberg SA (1981) In vitro growth of cytotoxic human lymphocytes. IV. Lysis of fresh and cultured autologous tumour by lymphocytes cultured in T cell growth factor. Cancer Res 41: 4420
10. Loveland BE, MacKenzie IFC (1982) Which T cells cause graft rejection? Transplantation 33: 217
11. Mazumder A, Grimm EA, Zhang HZ, Rosenberg SA (1982) Lysis of fresh human solid tumours by autologous lymphocytes activated in vitro with lectins. Cancer Res 42: 913
12. Ortaldo JR, Timonen TT, Vose BM, Alvarez JA (1982) Human natural killer cells as well as T cells maintained in continuous cultures with IL-2. In: Fefer A, Goldstein A (eds) The potential role of T cells in cancer therapy. Raven Press, New York, p 191
13. Pawelec GP, Hadam MR, Ziegler, A, Lohmeyer J, Rehbein A, Kumbier I, Wernet P (1982) Long-term culture, cloning and surface markers of mixed leukocyte culture-derived human T lymphocytes with natural killer-like cytotoxicity. J Immunol 128: 1892
14. Strausser JL, Mazumder A, Grimm EA, Lotze MT, Rosenberg SA (1981) Lysis of human solid tumours by autologous cells sensitised in vitro to alloantigens. J Immunol 127: 226
15. Vánky F, Klein E (1982) Specificity of auto-tumour cytotoxicity exerted by fresh, activated and propagated human T lymphocytes. Int J Cancer 29: 547
16. Vánky F, Stjernswärd J (1979) Lymphocyte stimulation (by autologous tumour biopsy cells). In: Herberman RB, McIntire KR (eds) Immunodiagnosis of cancer, part 2. Marcel Dekker, New York, p 998
17. Vánky F, Klein E, Stjernswärd J, Nilsonne U, Rodriguez L, Peterffy A (1979a) Human tumour-lymphocyte interaction in vitro. II. Conditions which improve the capacity of biopsy cells to stimulate autologous lymphocytes. Cancer Immunol Immunother 5: 63
18. Vánky F, Vose BM, Fopp M, Klein E (1979b) Human tumour-lymphocyte interactions in vitro. VI. Specificity of primary and secondary autologous lymphocyte-mediated cytotoxicity. J Natl Cancer Inst 62: 1407
19. Vánky F, Gorsky T, Gorsky Y, Masucci M-G, Klein E (1982) Lysis of tumour biopsy cells by autologous T lymphocytes activated in mixed cultures and propagated with T cell growth factor. J Exp Med 155: 83–95
20. Vose BM (1978) Cytotoxicity of adherent cells associated with some human tumours and lung tissues. Cancer Immunol Immunother 5: 173
21. Vose BM (1980) Specific T-cell-mediated killing of autologous lung tumour cells. Cell Immunol 55: 12
22. Vose BM (1982a) Quantitation of proliferative and cytotoxic precursor cells directed against human tumours: Limiting dilution analysis in peripheral blood and at the tumour site. Int J Cancer 30: 135
23. Vose BM (1982b) Separation of tumour and host cell populations from human neoplasms. In: Reid E (ed). Methodological surveys, vol II. Ellis Harwood, Chichester p 45
24. Vose BM, Bonnard GD (1982a) Specific cytotoxicity against autologous tumour and proliferative responses of human lymphocytes grown in interleukin 2. Int J Cancer 29: 33
25. Vose BM, Bonnard GD (1982b) Limiting dilution analysis of the precursor cells capable of specific cytotoxic or proliferative responses to human tumour cells and their expansion with lectin-free interleukin-2. In: Serron B (ed) Current concepts in human immunology and cancer immunomodulation. Elsevier Biomedical Press, Amsterdam, p 385
26. Vose BM, Bonnard GD (1983) Limiting dilution analysis of the frequency of human T cells and large granular lymphocytes proliferating in response to interleukin-2. I. The effect of lectin on the proliferative and cytotoxic activity of cultured lymphoid cells. J Immunol (in press)
27. Vose BM, Howell A (1983) Cultured human antitumour T cells and their potential for therapy. In: Herberman RB (ed) Clinical and basic tumour immunology. Martinus Hyhoff, The Hague (in press)
28. Vose BM, Moore M (1979) Suppressor cell activity of lymphocytes infiltrating human lung and breast tumours. Int J Cancer 24: 579
29. Vose BM, Moore M (1981) Cultured human T-cell lines kill autologous tumour. Immunol Lett 3: 237
30. Vose BM, Vánky F, Klein E (1977) Lymphocyte cytotoxicity against autologous tumour biopsy cells in humans. Int J Cancer 20: 512
31. Vose BM, Vánky F, Fopp M, Klein E (1978a) In vitro generation of cytotoxicity against autologous human tumour biopsy cells. Int J Cancer 21: 588

32. Vose BM, Vánky F, Fopp M, Klein E (1978b) Restricted autologous lymphocytotoxicity in lung neoplasia. Br J Cancer 38: 375
33. Vose BM, Gallagher P, Moore M, Schofield PF (1981) Specific and non-specific lymphocyte cytotoxicity in colon cancer. Br J Cancer 44: 846
34. Vose BM, Riccardi C, Marchmont RJ, Bonnard GD (1983) Clonal analysis of human natural killer cells. In: Herberman RB (ed) NK cells and other natural effector cells. Academic Press, New York, p 879
35. Zarling JM, Robins HI, Raich PC, Bach FH, Bach ML (1978) Generation of cytotoxic T lymphocytes to autologous human leukaemia cells by sensitisation to pooled allogeneic normal cells. Nature 274: 269

Received January 11, 1983