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Factors Influencing the Establishment of Tumour-infiltrating Lymphocyte Cultures from Human Breast Carcinoma and Colon Carcinoma Tissue

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Tumour-infiltrating lymphocytes (TIL) were obtained from breast and colon tumour tissue and cultured *in vitro* in the presence of recombinant human interleukin-2. Seven of 35 breast tumours and five of 41 colon TIL cultures were established *in vitro*: proliferation rates of greater than 10^3 were achieved. The cytotoxic capacity of these cells was determined against the cell lines K562 and SW742, and percentage cytotoxicity levels of greater than 97% and 79%, respectively, were seen. An inverse relationship between the ability of TIL to kill and their proliferative capacity was observed in all cultures. The prominant phenotype was CD3 positive, with greater than 55% of TIL expressing this antigen; there was no expression of CD16. The expression of CD56 and CD25 varied, being maximumly expressed on 64% and 38% of TIL, respectively. When greater then 90% of TIL expressed CD3, the ability of the culture to kill the target cell lines was low; only when there was an increase in the proportion of cells expressing CD56 and a decrease in the expression of CD3 was there high cytotoxicity. This study indicates that the TIL which proliferate *in vitro* in the presence of interleukin-2 are not necessarily the mediators of cytotoxicity.
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INTRODUCTION

THE STIMULATION of peripheral blood mononuclear cells (PBMC) with recombinant human interleukin-2 (rhIL-2) results in the appearance of lymphokine activated killer (LAK) cells capable of mediating non-MHC restricted cytotoxicity [1, 2]. These cells have been shown mainly to be CD3-negative, CD16-positive effectors, derived from large granular lymphocytes (LGL) or natural killer (NK) cells, together with a minor population of CD3 positive, CD16 positive/negative cells also possessing MHC unrestricted cytotoxicity [3]. Adoptive immunotherapeutic treatment of cancers using LAK effector cells and rhIL-2 has been undertaken, and the results show limited antitumour activity in patients with malignant melanoma, renal cell and colorectal carcinoma, giving clinical response rates of 35%, 21% and 17% respectively [4]: LAK cells in combination with rhIL-2 have not proved beneficial in the treatment of other human solid malignancies.

In an attempt to improve the clinical response of cancer patients to immunotherapy, lymphocytes isolated from the tumour mass, termed tumour infiltrating lymphocytes (TIL), have been assessed for the ability to promote tumour regression. Preliminary work in murine systems indicated that these cells

were 50–100 times more effective than LAK cells in mediating tumour regression [5]. TIL have also been evaluated in clinical trials in cancer patients, where 40% response rates have been achieved [6, 7]. Although TIL have been shown to localise in tumour tissue upon injection *in vivo* [8], the mechanism of action is not clear. Studies to characterise TIL, and modulate their activity *in vitro*, prior to the transfer of these cells back into the patient in order to improve the efficacy of therapy, are being carried out in many laboratories; however, to date such analysis of TIL from human tumours has been limited to renal cell carcinoma [9], squamous carcinoma of the head and neck [10], malignant melanoma [11] and carcinoma of the lung [12]. The present study was undertaken to characterise the phenotype, growth and antitumour cytotoxic activity of TIL isolated from human breast and colon carcinomas following *in vitro* culture with rhIL-2.

MATERIALS AND METHODS

Specimens

Primary tumour tissue was obtained at operation from 35 breast carcinoma and 41 colon adenocarcinoma patients. The mean ages were 63, 72 and 65 years for breast carcinoma patients and female and male colon carcinoma patients, respectively. Specimens were transported to the laboratory with minimum delay in sterile RPMI 1640 medium.

Culture medium

Culture medium consisted of RPMI 1640 medium, containing glutamine (final concentration 2 mmol/l), supplemented with 5% human autologous serum for breast cancer cells; and the same media with penicillin (50 U/ml), streptomycin (100 μg/ml), mycostatin (10 U/ml) and gentamycin (20 U/ml) for colon carcinoma cell preparations.

Recombinant human interleukin-2

RhIL-2 with a specific activity of 8.3×10^6 U/ml was kindly supplied by Glaxo (Geneva), and was used at final concentrations of either 500 or 1000 units/ml.

Preparation of tumour cell suspensions

Single cell suspensions from tumour tissue were obtained by enzymic digestion, as detailed by Ochoa [13]. Briefly, resected tumours were dissected to remove necrotic, fatty and apparent normal tissue, washed twice with phosphate buffered saline, and chopped into small pieces with two scalpel blades. The tumour fragments were treated with RPMI 1640 medium containing 200–300 U/ml collagenase IA, 0.02 mg/ml DNase I type IV and 0.01 mg/ml hyaluronidase VII (Sigma) and mechanically stirred at 37°C for 2 h. The resulting cell suspension was filtered through a steel mesh to remove undigested tumour fragments, and washed twice in RPMI 1640 medium. The cell preparations were layered onto a lymphocyte separation medium (Lymphoprep, Nygaard and Company) and centrifuged at 400 *g* for 35 min. The interface between the digest media and the separation medium containing the mononuclear cell population was collected, and washed three times before culture. A two-step density gradient consisting of 100% and 75% lymphoprep, was used in some experiments.

Growth of tumour infiltrating lymphocytes

Cell suspensions, containing TIL and tumour cells, were suspended in RPMI 1640 medium containing 5% autologous serum and antibiotics, counted and cell viability determined by trypan blue exclusion. Suspensions were diluted to a concentration of 1×10^6 viable cells/ml in the same medium, and 1 ml seeded into each well of a 24 well (16 mm diameter) plate (Falcon, Becton Dickinson). Initially, 1000 U/ml rhIL-2 was included in the culture medium; this was later reduced to 500 U/ml with no loss of activation. Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air and cell counts were recorded every 3–4 days. When the cell concentration exceeded 1×10^6 cells/ml, cultures were split at a ratio 1:2, by adding media to the cells and transfering 1 ml to a fresh well. Expanding cultures were later passaged into 75 cl tissue culture flasks (Falcon, Becton Dickinson), which were incubated in an upright position at 37°C in an atmosphere of 5% CO_2 in air.

Target cells

The erythroleukaemia suspension cell line K562 [14] was used as a target for measuring NK cytotoxicity, and SW742, an adherent colon adenocarcinoma cell line [15], was used as a target to measure LAK cell killing; the latter cell line is relatively insensitive to NK cytolysis but sensitive to LAK cells. Both cell lines were grown in RPMI 1640 medium containing 10% fetal calf serum and antibiotics, and routinely checked to ensure freedom from mycoplasma (Mycoplasma rapid detection system, Gen-Probe, California).

Chromium-51 (^{51}Cr) release assay

Target cells were radiolabelled with 3.7 MBq ^{51}Cr-labelled sodium chromate (Amersham, Aylesbury, UK) and incubated for 1 h at 37°C, in 5% CO_2 in air. Cells were washed in RPMI 1640 medium containing 10% calf serum, incubated for a further hour, washed again, counted and resuspended at 1×10^5 cells/ml.

Assays were carried out in triplicate, in round bottomed 96-well microtitre plates (Falcon Microtest III flexible assay plates, Becton Dickinson). Target cells (0.1 ml/well) were incubated with effector cells (0.1 ml/well) at set ratios. Plates were incubated at 37°C, in 5% CO_2 in air for 4 h: after this time, 100 μl of supernatant was harvested and the percentage cytotoxicity calculated as described previously [16].

Phenotypic analysis

Monoclonal antibodies (MoAb) were used to identify CD8 (T cytotoxic/suppressor), CD4 (T helper/inducer), CD3 (pan T), CD16 (NK/K), and CD 56 (NKH1) positive cells (Becton Dickinson, Oxford). Anti-CD25 was used to identify IL-2 receptor expression (DAKO, Buckinghamshire, UK). All MoAb were indirect conjugates except anti-CD16 which was a phycoerythrin (PE) conjugate. Approximately 5×10^5 cells in a 100 μl volume were incubated at 4°C for 20 min with each of the panel of monoclonal antibodies. The cells were washed in PBS then incubated at 4°C for 20 min with 100 μl of flurorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG. The Control samples were incubated with the FITC-conjugate alone. The cells were washed in PBS, then analysed by flow cytometry (FACS 420, Becton Dickinson, Sunnyvale, California), with an

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Table 1. Pathology of tumours and yield of tumour infiltrating lymphocytes (TIL) from specimens cultured in vitro *in the presence of rhIL-2 for greater than two weeks*

Patients (age, sex)	Tissue type	Differentiation	Cell recovery ($\times 10^6$/g tumour)
Breast tumour patients			
1 (90, F)	Invasive ductal adenocarcinoma	Poor	5.0
2 (44, F)	Adenocarcinoma (NOS)	Moderate	0.6
3 (41, F)	Invasive ductal adenocarcinoma	Poor	2.5
4 (65, F)	Adenocarcinoma (NOS)	Poor	1.0
5 (44, F)	Intraduct adenocarcinoma	Good	8.0
6 (72, F)	Invasive ductal adenocarcinoma	Poor	10.6
7 (33, F)	Invasive ductal adenocarcioma	Poor	2.6
Colon tumour patients			
8 (61, M)	Rectal adenocarcinoma, Dukes B	Moderate	6.5
9 (79, F)	Caecal adenocarcinoma, Dukes C	Poor	5.0
10 (65, M)	Sigmoid adenocarcinoma, Dukes C	Moderate	2.5
11 (65, F)	Rectal adenocarcinoma, Dukes C	Poor	8.0
12 (55, M)	Sigmoid adenocarcinoma, Dukes B	Moderate	13.6

NOS = Not otherwise specified.

excitation laser wavelength of 488 nm, suitable for FITC and PE. The results are expressed as a percentage of positive cells after subtraction of background fluorescence.

RESULTS

Cell yield following tissue digestion

Using the above protocols for digestion and isolation of TIL, the cell suspensions contained 60–96% viable mononuclear cells, with few, mainly non-viable, tumour cells: detailed phenotypic analysis at this stage was not possible due to low cell yields. A feature of this procedure was the range of cell recovery; colon specimens gave a mean of 9.2×10^6 cells/g wet weight (range 1.3–40.0) and breast specimens, a mean 3.0×10^6 cells/g wet weight (range 0.6–10.5). Results for those cultures which proliferated are shown in Table 1. No correlate could be established between cell recovery and the histology and differentiation status of the tumour.

A total of seven breast and five colon cultures were established in *in vitro* culture: cells from one out of 35 breast carcinomas and 12 out of 41 colon carcinomas failed to establish in culture due to bacterial contamination, and cultures established from approximately 20% of breast carcinomas and 17% of colon carcinomas failed to survive more than 14 days. Cells from a further 40% of breast carcinoma and 27% of colon carcinoma, from which low number of cells (less than 1×10^6) were isolated, did not proliferate. In those cultures maintained for longer than two weeks the fold increase in cell number achieved by 14 days, did not relate to the number of cells isolated or the initial seeding concentration.

In successful cultures, the cell concentration fell initially, but increased by the second week. The fold increase cell number was determined for those cultures which proliferated, and was shown to vary greatly between cultures. Table 2 shows the expansion of cultures determined by cell number and fold increase in cultures that were maintained for two or more weeks; the maximum period of *in vitro* expansion was 82 days and proliferation rates up to 2355 fold increase were observed.

Cytotoxicity of tumour infiltrating lymphocytes

TIL expanded *in vitro* were assayed for cytotoxicity against K562 and SW742, target cells for NK and LAK cell activity, respectively. Cytotoxicity values for the 12 cultures which were maintained for greater than two weeks are shown in Table 3. Background release of tumour targets used in these assays was always less than 15%. Significant killing was achieved by all but two of the breast TIL cultures, against both K562 and SW742 targets; the level of killing was similar for the two target cell lines. The cytotoxic capacity of colon TIL was less than that of breast TIL cultures using K562 target cells and the level of SW742 killing was approximately half that of K562. Positive controls (rhIL-2 stimulated normal donor PBMC) were included on all assay days. Breast tumour culture AH yielded cytotoxicity values of 2.2% and 2.4% compared to control stimulated normal donor PBMC cultures which gave 64.4% and 52.3% against K562 and SW742 tumour targets respectively. Similar control cytotoxicity values were obtained when cultures IH, AB and DH were assayed, showing that target susceptibility to PBMC LAK activity is independent of the TIL killing. Cytotoxic capacity did not correlate directly with cell proliferation (Table 3), and an inverse relationship between the capacity of the cell populations to proliferate *in vitro* and the ability of the cells to cause cell lysis was observed. Figure 1 shows representative killing of K562 and SW742 cells at different culture times, by TIL from two breast and two colon tumour cell populations, where high or low non-MHC restricted cytotoxicity and differing proliferative capacities are apparent.

Phenotype of TIL

Phenotypic analysis of cultured cells was undertaken where possible, and usually in parallel with cytotoxicity assays. The phenotypes of cells maintained in culture are shown in Table 3 in comparison with proliferation and cytotoxicity. In all cultures analysed, the prominant cell type was CD3 positive; this marker being expressed on 55 to 97% of the cells. The cytotoxic:helper (CD8:CD4) ratio varied between cultures; with one exception, a higher proportion of CD8 positive cells was observed. Expression of CD8 ranged from 29% to 87% and CD4 ranged from 5% to 67%. CD16 expression occurred on none of the cells. The expression of CD56 (NKH1) varied between cultures, being maximally expressed on 64% of assayed cells. Similarly,

Table 2. Proliferation of tumour infiltrating lymphocytes (TIL) cultured in the presence of rhIL-2. Proliferation was determined by viable cell counts (trypan blue exclusion)

	Proliferation (fold increase)							
	Day 0	Day 14		Day 21		Maximum		
	Cell no. ($\times 10^6$)	Cell no. ($\times 10^6$)	Fold increase	Cell no. ($\times 10^6$)	Fold increase	Cell no. ($\times 10^6$)	Fold increase	Day
Breast tumour patients								
1	13.5	50.0	3.7	121.5	9.0	156.6	11.6	16
2	1.6	2.4	0.6	11.4	7.1	150.4	94.0	30
3	6.8	10.1	1.5	25.7	3.8	68.2	10.1	23
4	1.0	11.9	11.9	79.2	79.2	734.4	734.4	28
5	1.0	ND	ND	ND	ND	ND	ND	ND
6	51.8	41.4	0.8	98.3	1.9	155.3	3.0	18
7	2.6	9.7	3.7	13.7	5.2	12.1	4.6	28
Colon tumour patients								
8	6.5	122.2	18.8	480.0	73.8	1755.0	270.0	24
9	5.0	25.0	5.0	48.0	9.6	11776.0	2355.2	82
10	2.5	14.0	5.6	29.0	11.,6	44.0	17.6	32
11	8.0	49.6	6.2	70.4	8.8	89.6	11.2	16
12	38.8	93.0	2.4	365.5	9.2	589.0	15.2	17

ND = Not determined.

Table 3. Functional and phenotypic characterisation of tumour infiltrating lymphocytes (TIL) cultured in vitro *in the presence of rhIL-2*

			Cytotoxicity*		Phenotype†				
	Day of assay	Proliferation (fold increase)	K562	SW742	CD 3	CD 4	CD 8	CD 56	CD 25
Breast patients									
1	13	4.2	2.2	2.4	95.8	67.8	29.0	ND	33.7
2	30	94.0	24.8	18.3	89.8	45.7	87.6	44.7	14.8
3	24	6.2	61.9	48.1	55.6	6.5	65.2	64.4	10.0
4	35	259.2	10.6	9.6	95.6	8.6	33.9	12.9	5.3
5	20	ND	57.1	60.9	ND	16.4	44.8	33.9	19.0
6	18	3.0	68.5	60.7	ND	ND	ND	ND	ND
7	26	5.2	71.3	79.5	ND	ND	ND	ND	ND
Colon patients									
8	24	270.0	13.2	6.6	97.1	10.2	72.5	15.9	38.0
9	34	124.8	4.0	6.6	ND	16.3	74.3	16.4	15.6
10	15	5.6	68.2	66.0	ND	ND	ND	ND	ND
11	17	11.2	50.9	26.7	74.5	5.4	52.7	33.2	0.7
12	17	15.2	97.6	49.7	80.0	21.9	33.8	29.3	19.8

ND = Not determined.
*Cytotoxicity of effector cells was assayed in a 4 h ^{51}Cr-release assay at an effector to target ratio of 5:1.
†Percentage of cells expressing surface antigens detected by flow cytometry.

the expression of the IL-2 receptor on TIL varied from 0.7% to 38% of cells expressing CD25 antigen.

DISCUSSION

Adoptive cellular immunotherapy in combination with cytokines has been investigated using both peripheral blood lymphocytes and TIL expanded *in vitro*. Thus using peripheral blood LAK and rhIL-2 therapy, observed responses were seen in 25% of 177 patients with a variety of malignancies [4]; in other studies responses were seen in 32% of 40 patients with various tumours [17], in 13% of 32 renal cell carcinoma patients [18] and in 47% of 29 patients with a variety of cancers [19]. Little additional benefit has been shown using rhIL-2 expanded TIL. 29% of patients with renal cell cancer and 23% of those with melanoma responded to TIL and continuous infusion of rhIL-2 [20], and 60% of 15 melanoma patients responded to therapy with TIL,

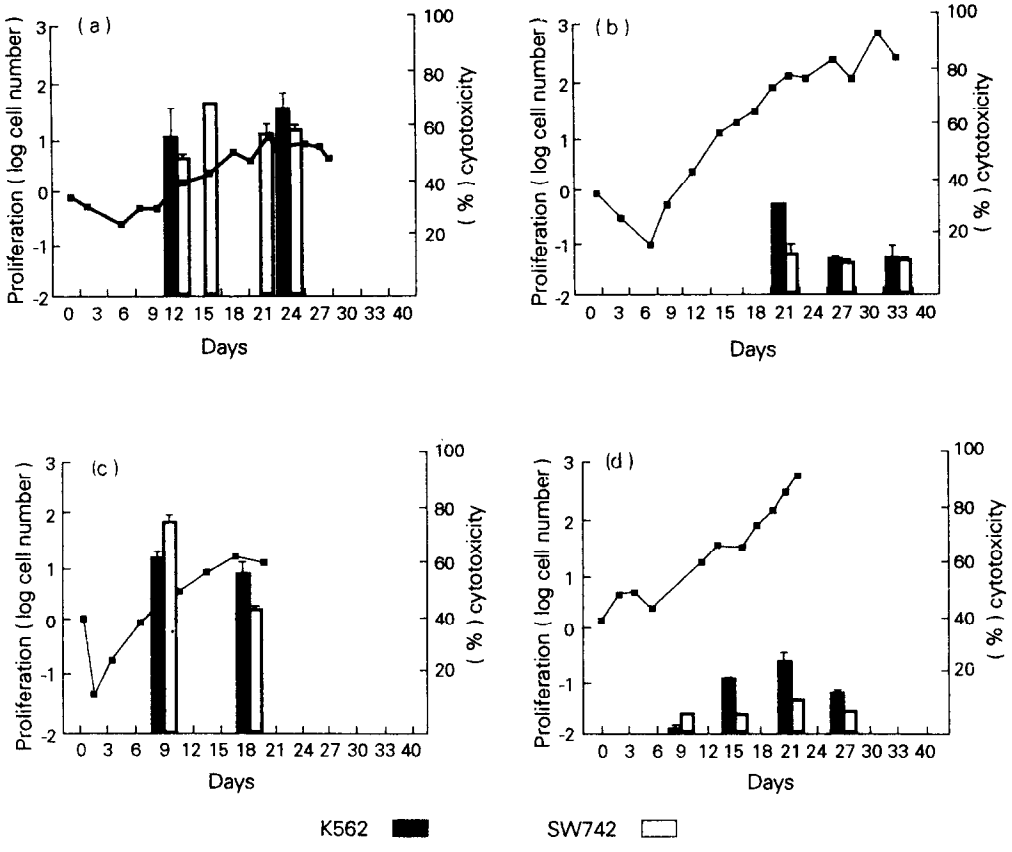

Fig. 1. Growth and antitumour cytotoxicity of representative IL-2 stimulated cultured tumour infiltrating lymphocytes (TIL) from 2 patients with breast carcinoma (A, B) and 2 patients with colon carcinoma (C, D). Proliferation of TIL was determined by viable counting and cytotoxicity was assessed in a 4 h ^{51}Cr-release assay against target cell lines K562 and SW742 at a 5:1 ratio.

rhIL-2 and cyclophosphamide [6]. In the present study we report some properties of TIL established from human breast and colon carcinoma tissue *in vitro*, since these techniques may have value in enhancing subsequent immunotherapies. Reports have indicated that the presence of tumour cells in lymphocyte cultures inhibit their ability to proliferate and generate cytotoxicity, and for this reason attempts were made to separate lymphocytes from tumour cells using two-step density gradients [21]. In general, an enrichment of lymphocytes or tumour cells at the appropriate interfaces was obtained, but the cell yield from many tumour tissues was low. TIL cultures prepared in this way did not proliferate, therefore in subsequent experiments tumour tissue digests were separated by one-step density gradients and tumour cells were present in the initial cultures. There was no correlation found between the size of the tumour and the number of cells isolated. This study compares to one which isolated TIL from 14 breast tumours, where the mean weight of tissue was 2.3 g (range 0.6–9.5 g), and the yield of cells obtained was 6×10^5 to 7×10^6 lymphocytes/g of wet tumour tissue [22]. In other studies, colon tumours of 0.7 to 9.6 g, produced 0.4 to 9.1 $\times$ 10^6 lymphocytes/g using a two-step gradient procedure [23]; and the yield of TIL from colon tumours of net weight 0.5–4.0 g using two-step density gradients gave good purity and viability, but as reported here, with losses in total cell numbers [24]. These results suggest that the present method using a one-step density gradient for separation gave the best yield.

An inverse relationship seen between proliferation and cytotoxicity was found in the present study; cultures achieving high proliferation were unable to mediate high tumour killing. Similar observations have reported in TIL from squamous cell carcinoma of the head and neck [10]. The ability of the cell populations to kill the two tumour cell targets showed distinct patterns of either high or low percentage cytotoxicity; this phenomenon may be related to the culture phenotype, or distortion of relative phenotypes during proliferation or passage. Analysis of the cytotoxic capacity of TIL in relation to their phenotype showed that for cultures consisting predominantly of CD3 positive cells, the killing ability was low; higher killing was observed when cultures expressed increased CD56 and reduced CD3. This observation is consistent with that of other workers [25, 26]. In the present study, high levels of cytotoxicity *in vitro* appears to be related to LGL-LAK cell cytotoxicity, though this may not relate to the ability of cells to mediate pronounced *in vivo* therapeutic effects; thus T cells have been shown to traffic more readily than LGL to tumour tissue [8], and may mediate direct tumour killing or indirect killing of cancer cells by secreting cytokines, which act directly on the tumour, or by recruiting other effector cells. The assumption is made that *in vivo* the tumour becomes enriched with lymphoid cells that have antitumour activity, and that this is of therapeutic benefit. Whilst evidence for T cell mediated elimination of solid tumours has been provided in murine models [5], the presence of specific tumour-reactive T lymphocytes at the tumour site in man has been difficult to demonstrate. It has been shown that some TIL cultured *in vitro* with rhIL-2 can lead to specific cytotoxic T cells directed against autologous tumour [11]. This rationale has led Yannelli and his colleagues, to expand in culture tumour-derived activated cells (TDAC) for immunotherapies; the clinical efficacy of these cells is currently being assessed [27]. *In vitro*

cultivation of TIL has not resulted in the generation of highly cytotoxic T cells with specificity against autologous tumours [9, 10,12,26,28], and clinical trials have yielded poor responses in the treatment of most tumours except in the treatment of malignant melanoma and renal cell carcinoma. This may be due to the immunogenic nature of these tumours, since specific T-lymphocyte responses have been reported, and have been shown to be mediated by the T cell receptor and to be MHC-restricted [11]. Much remains to be established before the value of TIL expanded and primed by treatment with cytokines or other immunomodulators can be seen to have any value in the treatment of malignant disease.

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