Eur J Cancer, Vol. 28A, No. 11, pp. 1910–1916, 1992.
Printed in Great Britain

0964–1947/92 $5.00 + 0.00

Tumour-infiltrating Lymphocytes in Cervical Carcinoma

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Tumour-infiltrating lymphocytes from cervical carcinomas were cultivated in the presence of interleukin-2. The majority of bulk cultures were cytotoxic against K562, Mel 1 and Caski cells. CD8$^+$ cells were the predominant subset in over 50% of cultures, with varying numbers of CD56$^+$ and CD25$^+$ cells. T cell clones were established from six tumour-infiltrating lymphocyte cultures and the majority exhibited non-MHC-restricted cytotoxicity. However, in one case cytotoxicity of several derived clones was limited to the autologous tumour and in another, to the autologous tumour and Caski cells. This study indicates that tumour-infiltrating lymphocytes can be amplified and cloned from cervical carcinoma biopsies in the presence of rIL2. Although the predominant cytolytic function is non-MHC-restricted, low autotumour cytotoxicity can be demonstrated at the clonal level. The nature of the antigen(s) recognised by T cells on autologous cervical carcinoma cells is unknown; the candidacy of human papillomavirus-related products requires investigation.
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INTRODUCTION

MANY HUMAN tumours elicit pronounced lymphocytic infiltrates which probably reflects, at least in part, immune recognition of tumour cells by the host immune system [1]. Several groups have shown that the presence of infiltrates in and around tumours of different histological type, including breast, colon and melanoma is a favourable prognostic sign [2–4]. These observations suggest that local immune mechanisms may play a protective role in retarding tumour growth and inhibiting the spread of malignant cells. Immunohistochemical analyses with monoclonal antibodies to leucocyte cell surface antigens have shown that the majority of tumour-infiltrating lymphocytes are T cells, with varying numbers of CD4 and CD8 cells [5, 6]. Few natural killer (NK), B cells and activated T cells expressing the interleukin 2R are found *in situ* [6].

Functional studies with freshly isolated tumour-infiltrating lymphocytes have shown depressed proliferative responses to mitogens and no or minimal NK activity and cytotoxic activity with cultured and fresh tumour cell targets [7]. However, with many human tumours insufficient material is available to analyse freshly isolated tumour-infiltrating lymphocytes. Methods have recently been developed that allow the isolation and propagation of tumour-infiltrating lymphocytes in the presence of interleukin-2 which allows their functional characterisation. Tumour-infiltrating lymphocytes from most solid human tumours proliferate in bulk culture under the influence of interleukin-2 and in the majority of cases, have been shown to exhibit non MHC-restricted cytotoxicity. Thus tumour-infiltrating lymphocytes isolated from lung carcinoma, squamous cell carcinoma of the head and neck, ovarian, breast and colon carcinoma exhibit varying degrees of cytotoxicity against autologous and allogeneic targets [8–11]. In contrast, tumour-infiltrating lymphocytes isolated from some malignant melanomas exhibit specific autologous tumour cytotoxicity [12]. The specificity of tumour-infiltrating lymphocyte populations may thus differ from tumour to tumour and probably also between different stages of the same tumour.

Little is known about the functional properties of tumour-infiltrating lymphocytes isolated from carcinoma cervix and their role in the antitumour response. These tumours are infiltrated with T lymphocytes [5], but no information is available regarding the functional characteristics of these lymphocytes and the relationship of the infiltrate to prognosis and survival.

One of the major risk factors in the development of carcinoma cervix is sexually transmitted infection associated with specific types of human papillomavirus. Human papillomaviruses 16, 18 and 33 are thought to be the active agents [13, 14]. Human papillomavirus DNA can be detected in up to 70% of cases of cervical cancer and although human papillomavirus infection alone is insufficient for cancer development, the immunogenicity of human papillomavirus-infected tumour cells and the immune response to them may be important in progression of cervical malignancy. Prior to an analysis of the specificity of tumour-infiltrating lymphocytes in cervical cancer for human papillomavirus antigens, this study was undertaken to ascertain whether tumour-infiltrating lymphocytes can be established in long-term culture in the presence of interleukin-2 from cervical tumour biopsies, and whether tumour-infiltrating lymphocytes in cervical cancer elicit an antitumour response and to analyse the nature of this response.

MATERIALS AND METHODS

Patients

Fresh tumour biopsy specimens were obtained from 27 patients with cervical carcinoma at EUA, prior to radiotherapy treatment at the Christie Hospital and Holt Radium Institute, Manchester. The ages ranged from 33 to 67 years. Histopathological data on each specimen are shown in Table 1.

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Received 5 May 1992; accepted 6 May 1992.

Table 1. Pathology of cervical tumours and days in culture of tumour-infiltrating lymphocytes (TIL) cultured in vitro *in the presence of rIL-2*

Patient (no.)	Age	Type	Differentiation	Stage	Days in culture of bulk TIL
1	56	S	P	IB	> 74*
2	54	S	W	IB	49†
3	33	S	P	IB	46
4	47	S	M	IB	63
5	43	A	W	IB	28
6	46	S	P	IB	28
7	33	S	P	IB	Poor growth†
8	50	S	P	IB	Contaminated
9	36	S	M	IB	No growth
10	52	S	M	IB	No growth
11	63	A	M	IB	No growth
12	38	S	M	IB	No growth
13	36	S	P	IIB	Contaminated
14	39	S	P	IIB	No growth
15	67	S	M	IIB	42
16	50	S	P	IIB	28†
17	45	S	P	IIB	No growth
18	57	S	M	IIB	61
19	50	S	M	IIB	56
20	50	S	M	IIB	56†
21	50	S	M	IIB	No growth
22	43	S	M	IIIB	102
23	56	S	W	IIIB	No growth
24	60	S	M	IV	83
25	55	S	P (small cell)	IVB	64†
26	44	S	M	IVB	91
27	44	S	P	IVB	47†

*Still growing at time of culture terminated.
†Clones obtained from bulk culture.
S, squamous cell carcinoma; A, adenocarcinoma. W, well differentiated; M, moderately differentiated; P, poorly differentiated.

Tumour cell suspensions and growth of tumour-infiltrating lymphocytes

Freshly excised tumour tissues were placed in sterile tissue culture medium with antibiotics. A piece of tissue (2 × 2 mm^2) were designated for immunohistology, if sufficient was available, and snap frozen in liquid nitrogen. The remaining tissue was minced with scalpels into pieces smaller than 1 mm^2 and the pieces subjected to enzymatic digestion with a mixture of collagenase 1 mg/ml and hyaluronidase 0.1 mg/ml in tissue culture medium (TCM) overnight. Following enzymatic digestion, suspensions containing tumour cells and tumour-infiltrating lymphocytes were centrifuged, washed twice in TCM and counted. In cases with sufficient material, a two-step density gradient consisting of 100% and 75% Lymphoprep was used to separate tumour cells and lymphocytes. These tumour cells were cryopreserved for use as target cells. Cell suspensions, containing tumour and tumour-infiltrating lymphocytes with no adjustment of tumour/lymphocyte ratios, were initiated with 0.5 – 1 × 10^6 cells/ml in TCM with 200 U/ml rIL-2 (Cetus) in 24 well Costar plates. Expanding cultures were transferred to 75^2 cm flasks and maintained at 10^6 cells per ml by adding fresh TCM and rIL-2 or subcultured as necessary. TCM consisted of RPMI 1640 supplemented with 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 μg/ml of streptomycin and 10% heat-inactivated fetal calf serum. T cell clones were obtained from bulk cultures by limiting dilution [15].

Immunohistology of tumour tissue and cultured cells

Cryostat sections of tumour tissues were cut at a thickness of 5 μm onto silicone-treated slides, air-dried overnight and fixed in acetone for 10 min. Tissues were stained with monoclonal antibodies using an immunoperoxidase technique [16]. The monoclonal antibodies used for lymphocyte staining were UCHTI (CD3), Dako T4 (CD4), Dako T8 (CD8), Act 1 (CD25; IL-2R β chain) and Leu 19 (CD56). W6/32 (anti-HLA class I) and CR3/43 (anti-HLA class II) were used to stain tissue sections. Slides were examined in a Leitz light microscope and the numbers of stained cells per high-power field (magnification ×400) were counted. 10 high-power fields were randomly selected and counted on every slide.

Cultured lymphocytes were phenotyped using the same panel of monoclonal antibodies and stained by an immunoalkaline phosphatase method on cytospin preparations [17].

Tumour cell targets

When sufficient cells were available after enzymatic digestion of tumour tissue and culturing of tumour-infiltrating lymphocytes, tumour cell suspensions were cryopreserved in 90% FCS and 10% dimethylsulphoxide. Immediately before cytotoxicity assays, tumour cells were thawed, washed in TCM, checked for viability and labelled with ^{51}Cr to serve as targets.

K562, an erythroleukaemic cell line was used as a target for measuring NK cytotoxicity, and Mel 1, an adherent melanoma cell line (established in this laboratory) and Caski, an adherent cervical carcinoma cell line, were used as targets to measure lymphokine-activated killer (LAK) cell killing; the latter two cell lines are resistant to NK cytolysis, but sensitive to LAK. These cell lines were maintained in culture in RPMI 1640 with 10% fetal bovine serum and antibiotics. Cells were subcultured as necessary and used in cytotoxicity assays.

Cytotoxicity assays

Fresh or cultured tumour cell targets were labelled with 3.7–7.4 × 10^6Bq of sodium chromate (18.5 × 10^6 Bq/ml) for 1 h at 37°C. Cells were then washed three times, resuspended in 10 ml TCM and incubated for a further 30 min. Cells were then washed twice, resuspended in fresh medium, counted and aliquoted at 2 × 10^3 targets per test-tube in LP2 tubes, into which the effector cells had previously been aliquoted. Assays were carried out in triplicate. The effector-to-target cell ratios ranged from 40:1 to 5:1. Tubes were centrifuged and then incubated at 37°C for 4 h. 100 μl of supernatant was harvested and the percentage cytotoxicity calculated as described [15].

RESULTS

Immunohistology

Immunoperoxidase staining with antibodies to T cell markers was performed in 14 tumours. In 3 cases, lymphocyte infiltration was rare, and in the remaining 11 tumours a varying no. of T cells was present. $CD3^+$ cells were present in the stroma surrounding the tumour and infiltrating the tumour mass. In the majority of cases the number of $CD8^+$ lymphocytes infiltrating the tumour mass was greater than that of $CD4^+$ lymphocytes. The mean T4/T8 ratio in the tumour was 0.9 (range 0.3–2.6) and in the stroma was 1.1 (range 0.5–1.9). Few cells appeared to be in the activated state as judged by the low nos. of lymphocytes expressing the interleukin-2R β chain.

Table 2. Immunohistologic analysis of lymphocytes infiltrating cervical carcinomas and MHC expression on tumour cells

	Tumour parenchyma					Stroma					Tumour cells	
Patient (no.)	CD3	CD4	CD8	Ratio CD4/CD8	CD25	CD3	CD4	CD8	Ratio CD4/CD8	CD25	MHC I	MHC II
1	19.2*	6.0	16.1	0.4	2.0	24.0	9.0	19.0	0.5	3.0	100†	100
2	16.3	10.0	15.5	0.6	1.2	24.1	20.1	9.2	2.2	3.1	100	100
3	5.4	3.7	2.9	1.3	0.8	17.5	12.0	9.6	1.3	1.0	100	100
4	6.6	4.4	6.1	0.7	0.7	10.4	6.1	4.7	1.3	1.1	100	100
13‡	27.3	13.4	17.0	0.8	8.0	36.0	22.0	28.0	0.8	3.6	100	100
14‡	16.6	4.2	12.1	0.3	1.8	19.0	9.0	9.3	1.0	4.2	100	100
15	30.0	13.0	25.0	0.5	2.0	18.0	13.0	7.0	1.9	4.0	100	50
16	14.9	10.1	11.0	0.9	1.6	23.0	11.0	15.0	0.7	3.3	100	100
17‡	2.9	11.3	2.1	0.6	1.3	10.7	8.3	6.5	1.3	3.1	100	100
18	8.2	5.1	5.1	1.0	1.0	12.8	6.2	9.3	0.7	1.4	100	100
22	12.1	8.2	9.4	0.9	8.0	23.7	18.5	20.0	0.9	10.8	100	75
24	22.5	12.1	18.5	0.7	5.0	44.0	30.0	30.2	1.0	8.7	100	50
25	3.7	4.7	1.8	2.6	0.2	10.1	6.0	4.6	1.3	1.9	70	10
26	5.3	3.0	3.7	0.8	1.1	24.1	11.1	20.0	0.6	2.0	100	75

*Cell counts per high-power field (10 HPF mag. × 400 counted per section).
† Percentage of tumour cells positive.
‡ Tumour-infiltrating lymphocytes failed to grow from these biopsy specimens.

Table 2 summarises the phenotype of infiltrating cells in 14 cases. All 14 tumours examined were MHC class I positive, the staining was uniform throughout the tumour in 13 cases and heterogeneous in 1 case. There was variable expression of MHC class II, 10–100% of the tumour mass being positive. There was no correlation between the intensity of infiltrate and MHC class I or MHC class II expression.

Expansion of tumour-infiltrating lymphocytes in rIL-2

After enzymatic digestion, single cell suspensions containing both tumour-infiltrating lymphocytes and tumour cells were cultured in the presence of interleukin-2. The cell suspensions contained varying numbers of mononuclear cells (20–80%) but detailed phenotypic analysis at this stage was not possible due to low cell yields. Long-term tumour-infiltrating lymphocyte cultures were successively established in 16 of 27 cases (Table 1). Cells from 2 cases (patients 8 and 13) failed to establish due to contamination and a further 8 cases failed to survive more than 2 weeks. A further case (patient 7) exhibited poor growth of tumour-infiltrating lymphocytes, though T cell clones were isolated from the culture at day 27. Under the culture conditions used, tumour infiltrating lymphocytes expanded *in vitro* from 28 to 100 days. The outgrowth of tumour-infiltrating lymphocytes did not correlate with cell recovery from the biopsy, stage of disease or phenotype of infiltrating cells (Tables 1 and 2).

Cytotoxicity of tumour-infiltrating lymphocytes grown in long-term cultures with rIL-2

Tumour-infiltrating lymphocytes serially grown as bulk cultures and expanded with rIL-2 were tested for cytotoxicity against cultured tumour cell targets. 13 bulk cultures exhibited cytotoxicity towards K562, (NK sensitive), Mel 1 (LAK sensitive), or Caski cell targets (LAK sensitive). Cytolytic activity was still apparent in seven cultures after 7 weeks of culture. 1 case showed cytotoxicity towards K562 cells only, 1 against Caski only (patient 25) and another case was negative to all three targets. Table 3 shows the cytotoxic profiles of representative cultures.

To evaluate further the cytolytic activities of uncloned tumour-infiltrating lymphocytes to autologous and allogeneic cervical tumour cells, cytotoxicity assays were performed against fresh cryopreserved tumour targets. Insufficient frozen tumour cells were available to test the cytotoxicity on serial samples, but

Table 3. Cytoxicity profiles of tumour-infiltrating lymphocyte populations obtained from cervical carcinomas and cultured in vitro *in the presence of rIL-2*

		% Cytotoxicity†					
Patient (no.)*	Days in culture	K562	Mel 1	Caski	tu 3‡	tu 4	tu 25
1	28	91	72	ND			
	48	71	61	ND			
	74	77	48	35			
3	40	52	45	32	1	3	8
4	18	90	55	73			
	57	58	17	19	3	1	12
5	28	81	65	60			
22	28	70	56	ND			
	96	67	53	44	5	13	22
24	21	70	37	ND			
	35	61	42	ND			
	83	79	45	17			
26	46	80	54	50			
	85	72	62	73	31	15	2

*Representative patients.
†Cytotoxicity of effector cells was assayed in a 4 h ^{51}Cr release assay at an effector to target ratio 25:1.
‡tu 3, tu 4 and tu 25 are cervical tumours from patients 3, 4 and 25, respectively.
ND,not determined.

Table 4. Cytoxicity and phenotype of bulk cultured tumour-infiltrating lymphocytes and representative clones from patient 16

	% Specific cytotoxicity*				Phenotype			
	Auto-tu	Caski	K562	Mel 1	CD3	CD4	CD8	CD56
Bulk (day 14)	ND	4	24	6	76†	40	32	10
Clone‡ 1	6	3	0	0	+	+	−	−
Clone 2	2	9	0	0	+	−	+	−
Clone 8	7	19	0	0	+	−	+	−
Clone 10	0	14	0	0	+	+	−	−
Clone 11	13	25	0	0	+	+	−	−
Clone 12	2	27	0	0	+	+	−	−
Clone 13	19	36	0	6	+	−	+	+
Clone 14	11	29	2	9	+	−	+	+

*Cytotoxicity of effector cells was assayed in a 4 h ^{51}Cr release assay at an effector to target ratio of 20:1.
†Percentage of cells expressing surface antigens detected by APAAP.
‡Clones were initiated at day 20 from the bulk culture.

Table 5. Cytotoxicity and phenotype of bulk cultured tumour-infiltrating lymphocytes and representative clones from patient 25

		% Specific cytotoxicity*				Phenotype			
		Auto-tu	Caski	K562	Mel 1	CD3	CD4	CD8	CD56
Bulk	day 29	ND	29	6	9				
	day 58	3.0	9.2	0	4.0	100†	43	52	5
Clone‡ 1		14	6	1	1	+	−	+	−
Clone 2		10	3	3	1	+	−	+	−
Clone 4		15	4	2	1	+	−	+	−
Clone 10		16	3	1	1	+	+	−	−
Clone 11		13	1	2	3	+	−	+	+
Clone 12		2	4	2	3	+	+	−	−

*Cytotoxicity of effector cells was assayed in a 4 h ^{51}Cr release assay at an effector to target ratio of 20:1.
†Percentage of cells expressing surface antigens detected by APAAP.
‡Clones were initiated on d34 from the bulk culture.
ND, not determined.

the results of one experiment with effectors and targets available at the time of assay are shown in Table 3. Autologous tumour targets (patients 3 and 4) were not lysed by bulk tumour-infiltrating lymphocyte populations, but low levels of cytotoxicity were observed to allogeneic targets (patients 4, 22 and 26).

For further analysis, bulk-cultured tumour-infiltrating lymphocytes were cloned by limiting dilution in the presence of allogeneic feeder cells. Clones were successively generated from six bulk tumour-infiltrating lymphocyte cultures. A variety of different cytotoxic profiles were represented. 10 clones were obtained from patient 2, all of which showed no cytotoxicity against K562 and Mel 1, although the bulk culture killed both these targets. Autologous tumour was not available from this case for analysis. Nine clones were generated from tumour-infiltrating lymphocytes of patient 7; eight of these were non-cytotoxic and one was cytotoxic to K562 (19%), Mel 1 (21%) and Caski (29%).

Of 14 clones generated from patient 16, three had low levels of cytotoxicity towards the autologous tumour (11–19%) and Caski cells (25–36%) and an additional three lysed Caski cells only. No cytotoxicity was detected against K562 or Mel 1 (representative clones are shown in Table 4). 20 clones were generated from patient 20, none showed cytotoxicity towards the autologous tumour, three exhibited low levels of cytotoxicity towards Caski, K562 and Mel 1 (data not shown). Table 5 shows the cytotoxicity of 6 representative clones from patient 25 together with that of uncloned lymphocytes. Of the 12 clones obtained, nine showed low levels of cytotoxicity (11–16%) to the autologous tumour. The clones did not show cytotoxicity towards K562, Mel 1 or Caski cells. However, the bulk culture showed cytotoxicity towards Caski. Seven of the eight clones generated from patient 27 exhibited cytotoxicity towards K562, Mel 1 and Caski (in excess of 20%), showing a similar profile to the bulk culture (Table 6). The eighth clone was non-cytotoxic.

Phenotypic analysis of tumour-infiltrating lymphocytes

Long-term cultures of tumour-infiltrating lymphocytes were analysed at different times during their culture (usually in parallel with cytotoxicity assays) for expression of lymphocyte markers. The results of representative patients are shown in Table 7. In all cultures examined, $CD3^+$ T lymphocytes were the predominant population, representing 81–100% of the cells.

The ratio of CD4:CD8 cells varied considerably between cultures and throughout the culture period. Expression of CD4 ranged from 16% to 77% and increased in long-term culture in patient 1 (16–21%) and patient 24 (51–75%). $CD8^+$ lymphocytes ranged from 29% to 87% and was the predominant subset in over 50% of the cultures (cf. ratio of CD4:CD8 lymphocytes seen on tissue sections). Cells expressing the interleukin-2R β chain (CD25 antigen) varied from 10% to 82% with increases observed in patients 1 and 22. These percentages are higher than those observed in the tissue sections. CD56 positive lymphocytes varied from 8% to 34% and generally increased with long-term culture. No correlations between phenotype of the bulk culture and cytotoxicity were noted, although patient 25 had low numbers of CD56 positive cells and the bulk tumour-infiltrating lymphocytes showed no cytotoxicity towards K562 and Mel 1 (Table 5).

The phenotypes of the clones obtained from patients 16, 25 and 27 are shown in Tables 4, 5 and 6, respectively. Clones generated from patient 16 were all $CD3^+$ and either $CD4^+$ or $CD8^+$. 2 of the $CD8^+$ clones expressed CD56. However, both $CD4^+$ and $CD8^+$ clones showed low levels of cytotoxicity

Table 6. Cytotoxicity and phenotype of bulk cultured tumour-infiltrating lymphocytes and representative clones from patient 27

		% Cytotoxicity*			Phenotype			
		Caski	K562	Mel 1	CD3	CD4	CD8	CD56
Bulk	day 25	50	81	65	95†	16	87	34
	day 42	ND	69	52	ND	ND	ND	ND
Clone‡ 1		55	79	78	+	−	+	+
Clone 2		57	88	69	+	−	+	+
Clone 3		42	77	59	−	−	−	+
Clone 4		45	81	66	−	−	−	+
Clone 8		7	5	6	+	−	+	−

*Cytotoxicity of effector cells was assayed in a 4 h ^{51}Cr release assay at an effector to target ratio of 20:1.
†Percentage of cells expressing surface antigens detected by APAAP.
‡Clones were initiated on day 25 from the bulk culture.
ND, not determined.

Table 7. Phenotypic profiles of tumour-infiltrating lymphocyte populations obtained from cervical carcinomas and cultured in vitro *in the presence of rIL-2*

Patient no.*	Days in culture	Phenotype					
		CD3	CD4	CD8	CD4/CD8 Ratio	CD25	CD56
1	28	84†	16	58	0.3	13	22
	48	80	21	71	0.3	66	23
3	40	99	77	29	2.7	82	10
4	57	90	44	60	0.7	19	9
5	28	87	55	30	1.8	10	15
22	28	81	37	55	0.7	20	23
	96	98	26	64	0.4	70	10
24	21	96	51	45	1.1	29	8
	35	97	49	38	1.3	20	11
	83	95	75	29	2.6	12	28
26	85	98	14	84	0.2	12	22

*Representative patients.
†Percentage of cells expressing surface antigens detected by APAAP.

towards the autologous tumour and Caski cells. All the clones obtained from patient 25 were $CD3^+$, three expressed $CD4^+$ and nine were $CD8^+$. One clone also expressed CD56, and both $CD4^+$ and $CD8^+$ clones exhibited low levels of cytotoxicity to the autologous tumour. All seven cytolytic clones generated from patient 27 expressed CD56, five also expressed $CD3^+$ $CD8^+$ but two were $CD3^-$ $CD8^-$. The non-cytolytic clone 8 was $CD3^+$, $CD8^+$, $CD56^-$.

To determine the phenotype of effector cells in the NK and LAK cells assays, in 2 patients the bulk cultures were sorted into $CD56^+$ and $CD56^-$. The purity of each population was over 95%. In patient 24, only the $CD56^+$ cells showed any cytotoxicity towards K562 and Mel 1. However, in patient 27, low levels of cytotoxicity were observed to Mel 1 (NK resistant, LAK sensitive) in $CD56^-$ cells (Table 8).

DISCUSSION

The present study was undertaken to address the possibility of expanding lymphocytes from cervical carcinoma biopsy samples in long-term culture in rIL-2 and examining their phenotypic and functional characteristics. To our knowledge this is the first report that T cell lines can be established from cervical carcinoma biopsy specimens and the first evidence that T cell clones can be generated that recognise autologous cervical tumours.

Table 8. Cytotoxicity of $CD56^+/CD56^-$ sorted tumour-infiltrating lymphocytes against K562 and Mel 1 targets

		% Cytotoxicity*	
		K562	Mel 1
Patient 24 (day 39)	$CD56^+$	47	34
	$CD56^-$	7	−1
	Unsorted	61	42
Patient 27 (day 47)	$CD56^+$	66	41
	$CD56^-$	6	18
	Unsorted	38	28

*Cytotoxicity of effector cells was assayed in a 4 h ^{51}Cr release assay at an effector to target ratio of 20:1.

T cell lines were obtained from 16 specimens. Functional analysis revealed different cytotoxic profiles in that the majority exhibited LAK or non-MHC-restricted effector cell activity, whereas 1 line showed lysis of an allogeneic carcinoma cervix cell line only, and another was non-cytotoxic. No cytolytic activity was detected to autologous tumour targets assayed with T cell lines. However, in the majority of cases, autologous tumour cell targets were not available and we could not ascertain if the bulk cultures contained tumour specific effector cells in addition to non-MHC-restricted effector cells. Lymphoid cells accumulating at a tumour site could potentially represent a heterogeneous mixture of effector cells, including specific cytotoxic T lymphocytes, NK and LAK cells. The composition of the starting lymphoid population and method of stimulation may determine the phenotype and specificity of the cell generated [18]. The presence of tumour cells may act as a further *in vitro* stimulus for propagation of specific CTL [19, 20] or may even exert a suppressive effect [7]. In this study exogenous interleukin-2 was added to the cultures to maintain them in culture and this may preferentially stimulate not only specific activated cells accumulated at the tumour site but also LAK precursor cells. Interleukin-2-activated tumour infiltrating lymphocytes frequently show non-MHC-restricted cytotoxicity [9, 10]. However, the nature of the effector cell remains unclear. The majority of interleukin-2-activated PBMC that kill various tumour cells are derived from NK cells and are $CD3^-$. Few cells showing NK phenotype are found in tumours, although it is possible that both T cells and NK cells are involved in non-MHC-restricted killing.

In a study of ovarian tumour infiltrating lymphocytes cultured in interleukin-2, the effector cells exhibiting cytotoxicity of autologous and allogeneic ovarian tumours expressed CD56, and were either $CD3^+$, $CD56^+$ or $CD3^-$, $CD56^+$ [10]. The $CD3^+$, $CD56^-$ population, which was the main population in their cultures, did not mediate lysis. Cell sorting of two bulk tumour-infiltrating lymphocyte cultures in this study followed by cytotoxicity against cultured cell lines showed that the $CD56^+$ phenotype was involved in non-MHC-restricted killing, but double staining was not performed to assess the role of CD3 on these effectors. However, the clones obtained from patients 20 and 27 that exhibited LAK were either $CD3^+$, $CD8^+$, $CD56^+$ or $CD3^-$, $CD8^-$, $CD56^+$. Both phenotypes have been described in mediating MHC-unrestricted cytotoxic activity [18, 21].

Bulk tumour-infiltrating lymphocyte populations in this study showed a heterogeneous mixture of phenotypes. In some cultures $CD4^+$ lymphocytes predominated and in others $CD8^+$ cells. The two cultures exhibiting no cytotoxicity towards K562 and Mel 1 had approximately equal numbers of $CD8^+$ and $CD4^+$ cells, and in 1 case (patient 25) only low numbers of $CD56^+$ cells. In these cases the tumour infiltrating lymphocytes may have comprised a select population of lymphocytes with antitumour activity but insufficient autologous tumour was available to evaluate this from the bulk culture. If a specific CTL subpopulation is present whose activity is diluted out by other effectors, clonal analysis or long-term culture of tumour-infiltrating lymphocytes may be able to demonstrate these effectors. LAK cells do not grow beyond a few weeks in culture and an increase in specific cytotoxicity may represent an outgrowth of specific CTL and a demise of LAK cells. Topalian *et al.*[12] showed that four of 14 tumour-infiltrating lymphocyte cultures

derived from human melanomas grown in the presence of interleukin-2 manifested highly specific lytic activity as a function of time in culture. These cultures failed to lyse autologous normal cells or allogeneic cells and provided evidence for tumour specific MHC-restricted cytotoxic cells.

In this report, as one bulk culture showed selective lysis of a cultured cervical carcinoma cell line, it was decided to clone out tumour-infiltrating lymphocyte populations to try to expand any specific antitumour effector cells that may be present. Clones were generated from 6 cases and different cytotoxic profiles were observed. Autologous tumour cells were only available for 3 cases, but in 2 of these, patients nos. 16 and 25, low levels of cytotoxicity were observed to the autologous tumour. Three clones from patient 16 recognised antigens on the autologous tumour and on Caski cells, whereas three other clones recognised structures on Caski cells but no corresponding structures on the autologous tumour. These preliminary data suggest that lymphocytes from patients with cervical carcinoma have the potential to recognise more than one cell surface structure, though the nature of the recognition, particularly in the case of the Caski cells, is presently unknown.

The low levels of killing of the autologous tumour cells could indicate heterogeneity amongst the tumour cell population with only a small number of the target cells expressing an antigen recognised by specific CTL. Ioannides *et al.* [19] isolated tumour specific CTL lines and clones from lymphocytes infiltrating an ascitic ovarian tumour. Analysis of the reactivity of the clones against cloned autologous tumour cells revealed differences in the susceptibility to lysis of the autologous tumour cell clones and at least three different CTL-defined epitopes were described. Recent reports of studies with melanoma have also shown differential expression of determinants recognised by cytolytic T cells amongst melanoma cells [22].

At present, there is no information about the specificity of recognition by lymphocytes infiltrating cervical tumours or about the nature of the target antigen. Up to 70% of cervical carcinomas contain integrated human papillomavirus DNA and it is conceivable that CTL recognise human papilloma virus peptides in association with MHC molecules. Recent reports have described naturally processed viral peptides that are recognised by specific CTL in the cases of influenza virus and vesicular stomatitis virus [23, 24]. Caski cells contain human papillomavirus 16 DNA and at least one of the two cases showing autologous tumour cytotoxicity was human papillomavirus 16 positive (the other case has not been human papillomavirus-tped). Unpublished observations showed prolilferation of T cell clones from patient 16 to human papillomavirus 16 LI fusion protein as well as to autologous tumour and Caski cells. Further work is required to answer the question whether tumour infiltrating lymphocytes obtained from cervical carcinomas can recognise human papilloma virus antigens.

Analysis of the tumour cells revealed uniform MHC class I expression except in 1 case and either uniform or heterogeneous class II expression. Connor and Stern [25] have previously shown downregulation of MHC class I in a proportion of cervical tumours using monoclonal antibodies to monomorphic determinants. However, whether this phenomenon would effect the type of effector cell mediating immune recognition of the tumour remains to be elucidated. Glew *et al* [26], in a study from this laboratory on a larger series of cervical neoplasms, have shown that over 50% of squamous cell carcinomas express MHC class II products uniformly and that over 80% had some degree of class II expression, unlike the epithelium from which they are derived which is MHC II-negative. The significance of class II expression on human tumours appears to vary with histological type; in laryngeal and breast carcinomas it indicates a favourable prognosis [27] and this may also be the case in colon carcinoma where class II expression is proportionately greatest for Dukes' A compared with Dukes' B and C [16]. By contrast in metastatic melanoma MHC class II expression appears to have a negative influence on survival [28].

Analysis of the genotype of tumour-infiltrating lymphocytes provides information regarding the clonality of the cell population. DNA was prepared from a small number of bulk tumour-infiltrating lymphocyte populations, digested with EcoRI and analysed by Southern blot hybridisation with probes for TCRβ and γ1 genes. In two samples particular TCRβ-chain rearrangements were not detected, which indicates that these lymphocyte populations were polyclonal. In contrast in three samples, TCR gene rearrangements were detected consistent with the presence of oligoclonal populations of cells. In 2 cases, these involved two different rearrangements to Cβ1 and in a third case rearrangement to Jγ1 (unpublished observations). There are two explanations for oligonality of *in vitro* expanded tumour-infiltrating lymphocytes. They may represent outgrowth of specific antigen-primed T cells from the tumour site or alternatively they could represent an *in vitro* artefact with selective expansion of specific cells in the presence of interleukin-2. Evidence for the former hypothesis is suggested by the observation that in 1 case (patient 25) which showed rearrangement of the Cβ1 gene, analysis of cytotoxicity of the bulk culture and T cell clones revealed low levels of cytotoxicity to the autologous tumour and Caski cells but no cytotoxicity towards an NK-sensitive target.

Gervois *et al.* [29] found identical configuration of functional TCRβ genes in 'bulk culture' and 4 $CD8^+$ clones isolated from tumour-infiltrating lymphocytes from a melanoma patient, all of which were strongly cytotoxic for the autologous tumour. In addition, Yoshino *et al.*[30] have recently shown oligoclonal T lymphocytes infiltrating human lung cancer tissues. These observations provide further evidence for the existence of tumour specific immune responses.

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Acknowledgements—This work was supported by the Cancer Research Campaign.

Eur J Cancer, Vol. 28A, No. 11, pp. 1916–1920, 1992.
Printed in Great Britain

0964–1947/92 $5.00 + 0.00

Feature Articles

Phenotypic Features of Stromal Cells in Normal, Premalignant and Malignant Conditions

Annette Schmitt-Gräff and Giulio Gabbiani

INTRODUCTION

THE DYNAMIC cooperation between organotypic cell populations and their mesenchymal stroma is a common theme in embryonic development, tissue repair throughout life, and malignancy. Evidence is accumulating that extracellular matrix components, soluble cellular products and direct cell-to-cell contacts constitute microenvironmental signals implicated in tissue morphogenesis as well as in carcinogenesis [1–6]. Local perturbations in the tumour–host interactions are considered to play a key role in cancer development, angiogenesis, invasion and metastasis [7–12]. Moreover, it is well recognised that the bone marrow stroma is of crucial importance for the coordinated developmental pathway of the haematopoietic stem cells and their progeny [13], while leukaemic populations escape such positive and negative regulatory influences [14, 15]. The purpose of this review is to briefly highlight the phenotypic features of stromal fibroblasts in different types of premalignant and malignant situations. Previous well-referred reviews have covered the area of stromal regulation of epithelial functions [9, 16, 17].

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Received 15 Apr. 1992; accepted 12 May 1992.

HETEROGENEITY OF FIBROBLASTIC CELLS IN NORMAL AND PATHOLOGICAL TISSUES

The information gathered from numerous *in vivo* and *in vitro* studies supports the view that fibroblastic cells present in normal