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Lymphocytes infiltrating colorectal cancer have low proliferative capacity but can secrete normal levels of interferon γ

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Abstract Significant numbers of infiltrating mononuclear cells are commonly observed in solid tumours, although their role in restricting tumour growth is not clear. Tumour-infiltrating lymphocytes (TIL) from 38 patients with colorectal cancer, in parallel with peripheral blood lymphocytes (PBL), were assayed to determine their ability to proliferate in response to concanavalin A (ConA), interleukin-2 (IL-2), ConA + IL-2, phorbol 12-myristate 13-acetate (PMA) + ionomycin (IOM), and staphylococcal enterotoxin B (SEB). These reagents were selected to give a range of weak to strong proliferative responses either via or independent of the T cell receptor. Proliferation of TIL was significantly lower than that of PBL in all cultures: ConA ($P < 0.001$), IL-2 ($P = 0.002$), ConA + IL-2 ($P < 0.001$), PMA + IOM ($P < 0.001$), SEB ($P = 0.002$). In addition to the low proliferative capacity of TIL, production of cytokines by TIL may also play a role in control of tumour growth. We have assayed IFNγ production in the supernatants from 16 paired TIL and PBL cultures, and tumour necrosis factor α (TNFα) in 6 paired cultures. TNFα concentrations were significantly lower in TIL cultures than in PBL cultures stimulated with ConA ($P < 0.05$), but no different in control or IL-2 stimulated cultures. IFNγ levels did not significantly differ between PBL and TIL cultures, indicating that despite the restricted proliferative capacity of TIL, these cells remain capable of secreting significant amounts of IFNγ.

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Introduction

Malignant tumours are thought to arise by the accumulation of multiple genetic changes. Such a process will inevitably give rise to altered patterns of protein production and therefore the potential to stimulate an immune response [30]. In man, melanoma appears to be particularly immunogenic and as many as five HLA-class-I-restricted tumour antigens have been described (reviewed in [24]). In the more common solid tumours no tumour-specific antigen gene has yet been cloned; however, data exist in several tumour types implying expression of tumour-specific antigens [7,9,33]. The development of an effective immune response will depend critically on the capacity of tumour cells to present antigens, and this will be in part determined by a requirement for accessory molecules such as intercellular adhesion molecule-1 (ICAM-1) and B7 on the target and/or accessory cells. Appropriate cytokine production by activated lymphocytes and monocytes can then sustain the response. It is thought that, in the absence of this "complete" signal for activation, lymphocytes may become anergised [3]. Inadequate expression of major histocompatability complex (MHC) human leukocyte antigens (HLA-ABC and HLA-DR) required for antigen presentation to T cells and ICAM-1, or production of immunosuppressive agents such as transforming growth factor β (TGFβ) [29], prostaglandins [20] or interleukin-10 (IL-10) [32], may favour anergy in lymphocytes that recognise tumour antigens. We have compared in this study the capacity of tumour-infiltrating lymphocytes (TIL) and peripheral blood lymphocytes (PBL) from colorectal cancer patients to proliferate and produce interferon γ (IFNγ) and tumour necrosis factor α (TNFα) in vitro. We have chosen to concentrate on IFNγ production

because of its important immunoregulatory role. IFNγ, produced by activated T cells and natural killer (NK) cells, induces expression of accessory molecules such as ICAM-1 and its ligand leukocyte function antigen-1 (LFA-1), and enhances antigen presentation by increasing cell-surface expression of HLA-ABC, HLA-DR and up-regulating expression of the molecules involved in antigen processing [17]. In addition to its pro-inflammatory properties, TNFα can also enhance the expression of HLA and adhesion molecules in some cells, and synergises with IFNγ in their induction [19].

Materials and methods

Patients

Tumour material was obtained from patients undergoing resection of colorectal cancer at the Walsgrave Hospital, Coventry. Of 45 patients entered into this study, TIL from 38 were used in proliferation assays. There were 26 men and 12 women with an average age of 69.1 years. Four patients had early cancers (Dukes' A), 32 had advanced cancer (Dukes' B or C) and 1 had metastatic disease (Dukes' D). There were 37 adenocarcinomas, 1 of which was recurrent disease, and 1 adenoma. Five tumours were poorly differentiated, 29 moderately differentiated, 2 well differentiated, 1 mucoid and 1 adenoma. This work has been approved by the Coventry Ethical Committee.

Preparation of PBL

PBL were isolated from 20 ml heparinised blood by centrifugation over a density gradient (lymphoprep, Nycomed Pharma AS, Oslo, Norway) and incubated overnight with enzymes alongside the tumour (see below), so as to provide comparable samples. We found both viability and proliferation to be unchanged by this overnight incubation at room temperature.

Preparation of TIL

Tumour material was obtained immediately after resection and placed in cold culture medium for transport to the laboratory. The tumour piece, 1–3 cm in diameter, was minced with scalpels and disaggregated overnight at room temperature in culture medium containing 1 mg/ml collagenase type II (Worthington Biochemical Corporation, N.J., USA) 0.02 mg/ml DNase 1 type II (Sigma, Poole, UK) and 0.01 mg/ml hyaluronidase (Boehringer-Mannheim GmbH, Germany). The cell suspension obtained was layered over Lymphoprep, and centrifuged to isolate lymphocytes and tumour cells at the interface. After washing, the cells were centrifuged over 1.055 g/ml Percoll (Sigma, Poole, UK) to separate lymphocytes from the tumour cells and any debris. In tests with PBL this purification step was shown to have no effect on results obtained in proliferation assays, and essentially all of the PBL pelleted through this density of Percoll. In 19/38 experiments sufficient TIL were obtained to allow flow-cytometric analysis (Becton-Dickinson FACStar) of cells stained with flourescent antibodies to CD3 and CD4 (Becton-Dickinson, Cowley, UK). This confirmed the enrichment of mononuclear cells in TIL preparations.

Culture media

PBL and TIL were cultured and washed in HEPES-buffered RPMI medium (Sigma) containing 10% human AB serum (Sigma) and supplemented with 2 mM glutamine, 20 μM 2-mercaptoethanol, penicillin (60 μg/ml), streptomycin (100 μg/ml), fungizone (25 μg/ml; Gibco BRL, Paisley, Scotland), and gentamycin (50 μg/ml; Sigma). Only *Mycoplasma*-screened and endotoxin-tested tissue-culture media were used.

Proliferation assays

All assays were performed in triplicate in 96-well round-bottomed microtitre plates. The cell numbers added to each well varied between experiments depending on the numbers of TIL recovered, but numbers of TIL and PBL were kept equal for individual patients. Between 2×10^4 and 2×10^5 cells/well were incubated with medium alone or with one of the following: concanavalin A (ConA; 5 μg/ml), IL-2 (25 U/ml), ConA plus IL-2, phorbol 12-myristate 13-acetate (PMA; 10 ng/ml) plus ionomycin (IOM; 5 μg/ml), and staphylococcal enterotoxin B (SEB; 10 μg/ml) (all Sigma). Cells were cultured for 74 h at 37 °C, then for a further 18 h following addition of 1 μCi/well tritiated thymidine. Cells were harvested onto glass-fibre filters and the amount of radioisotope incorporated into DNA was measured by scintillation counting. Results are expressed as the stimulation index (SI), given as:

SI = mean radioactivity (cpm) of stimulated cells/

mean radioactivity (cpm) of control cultures.

Cytokine ELISA

Cytokine concentrations were measured in supernatants from the proliferation assays at 92 h when the assay was harvested. A 70- to 100-μl sample was removed from each well and supernatants from pooled triplicates were centrifuged to remove debris and stored at −80 °C until assayed.

Measurement of cytokines was carried out in duplicate using pre-prepared kits for IFNγ (Intertest-γ, Genzyme, Mass., USA) and TNFα (Predicta kit, Genzyme, Mass., USA). Results are expressed in pg $(10^6 \text{ cells})^{-1} \text{ml}^{-1}$ as determined by the standards provided with each kit.

Statistics

Results were compared by Wilcoxon signed-rank pairs test.

Results

Tumour-infiltrating lymphocytes

TIL populations were obtained from 45 tumours and 38 of these were used in proliferation assays alongside the patients' PBL, the remainder were not used either because there were insufficient TIL, or because the culture was contaminated. The numbers of TIL recovered from the tumour sample varied from 1.2×10^5 to 67.0×10^5 (mean = 17.6×10^5).

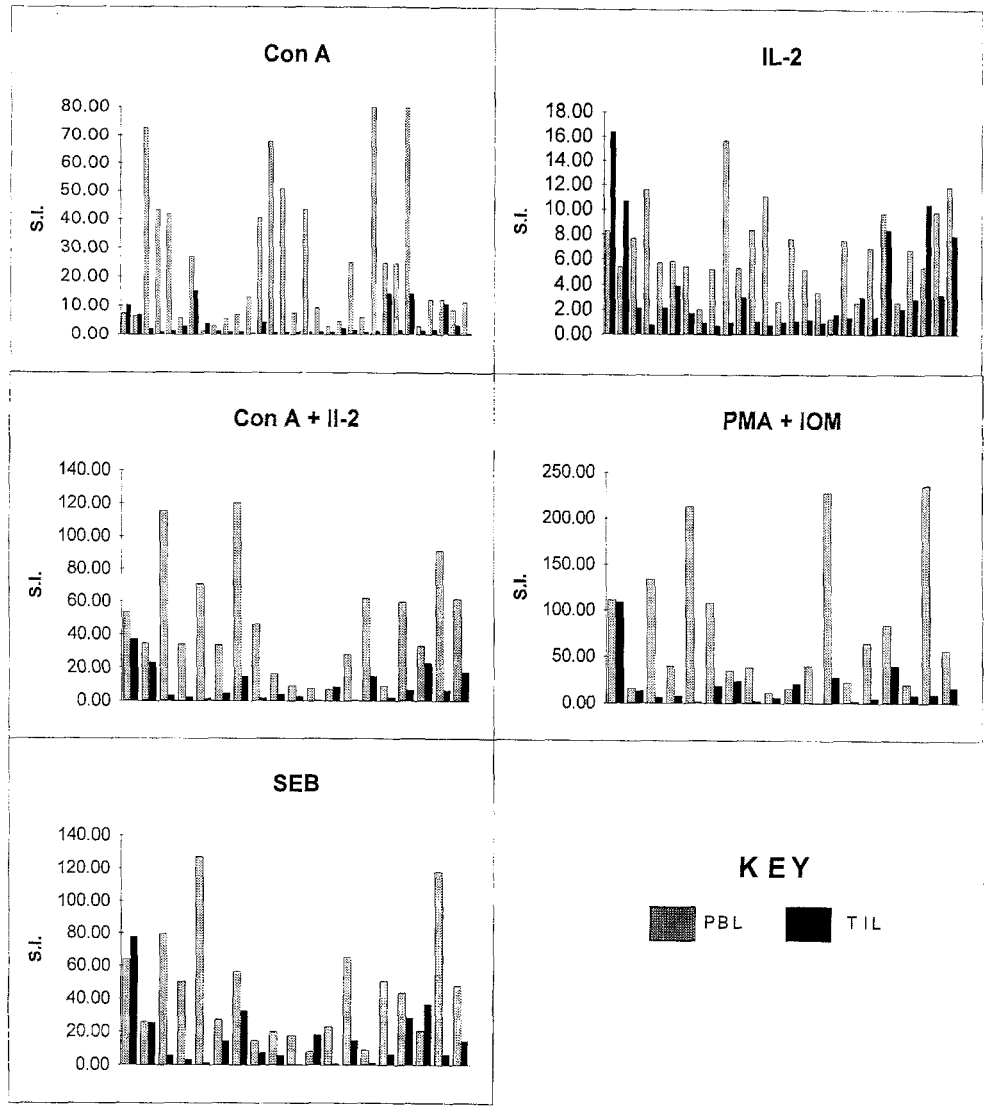

Fig. 1 Proliferation assays. Results are shown for each pair of tumour-infiltrating lymphocytes (*TIL*) and peripheral blood lymphocytes. Proliferation was measured by incorporation of tritiated thymidine: *S.I.* mean radioactivity (*cpm*) in treated cultures/mean radioactivity (cpm) in unstimulated cultures. Each bar indicates the mean of three replicates. *ConA* concanavalin A, *IL-2* interleukin-2, *PMA* phorbol 12-myristate 13-acetate, *IOM* ionomycin, *SEB* staphylococcal enterotoxin B

Proliferation assays

Results of proliferation assays are shown in Fig. 1 for each PBL/TIL pair. In all cases PBL responded significantly better than TIL, as determined by the Wilcoxon signed-rank pairs test. Ratios of the mean SI for PBL compared to TIL were as follows: 4.61 ($P < 0.001$) for ConA, 2.22 ($P = 0.002$) for IL-2, 5.0 ($P < 0.001$) for ConA plus IL-2, 4.32 ($P < 0.001$) for PMA + IOM and 2.75 ($P = 0.002$) for SEB.

Cytokine production

In a preliminary experiment we found that none of the control cultures or those treated with ConA or IL-2 contained detectable IFNγ. For this reason we subsequently concentrated on those cultures with PMA + IOM or SEB. Supernatants from 16 proliferation assays were available for IFNγ detection by ELISA. Results are shown in Table 1. IFNγ production for PBL was in the range 0–368 pg $(10^6 \text{ cells})^{-1}\,\text{ml}^{-1}$ in unstimulated cultures (mean = 23.75 ± 91.85), 57.25–2744 pg $(10^6 \text{ cells})^{-1}\,\text{ml}^{-1}$ for PMA + IOM (mean = 561.47 ± 646.89) and 0–104 pg $(10^6 \text{ cells})^{-1}\,\text{ml}^{-1}$ for SEB (mean = 15.53 ± 28.5). For TIL the corresponding figures were 0 pg $(10^6 \text{ cells})^{-1}\,\text{ml}^{-1}$ for unstimulated cultures, 0–1304 pg $(10^6 \text{ cells})^{-1}\,\text{ml}^{-1}$ for PMA + IOM (mean = 441.31 ± 361.14) and 0–492 pg $(10^6 \text{ cells})^{-1}\,\text{ml}^{-1}$ for SEB (mean = 103.78 ± 174.85). There was no statistical difference in IFNγ concentrations between cultures of TIL and PBL, although in SEB-treated cultures the IFNγ levels appeared to be higher in the TIL cultures. When proliferation (SI) and IFNγ concentration were compared in by regression analysis in cultures treated with PMA + IOM or SEB, we found no correlation between them for either PBL or TIL (Fig. 2). In six experiments

Table 1 Interferon γ concentration in paired cultures of tumour-infiltrating lymphocytes (*TIL*) or peripheral blood lymphocytes (*PBL*) stimulated with phorbol 12-myrystate 13-acetate + ionomycin or staphylococcal enterotoxin B (*SEB*). Values are means ± SD of duplicate samples

	Interferon concentration [pg $(10^6$ cells$)^{-1}$ml^{-1}]					
Experiment	PBL control	TIL control	PBL PMA + IOM	TIL PMA + IOM	PBL SEB	TIL SEB
LW35	0	0	154.8 ± 17.4	482.0 ± 5.8	0	196.0 ± 17.4
LW31	368.0[a]	0	992.0 ± 719.6	688.0 ± 63.8	32.0 ± 5.8	352.0 ± 5.8
LW29	0	0	90.0 ± 87.0	852.0 ± 40.6	24.0 ± 40.7	0
LW27	0	0	524.0 ± 5.8	1304.0 ± 29.0	8.0 ± 11.6	492.0 ± 5.8
LW26	12.0 ± 0.0	0	323.0 ± 5.8	217.0 ± 17.4	53.0 ± 23.2	49.0 ± 2.9
LW22	0	0	57.3 ± 5.8	50.0 ± 0.2	27.5 ± 5.8	23.5 ± 0.0
LW12	0	0	368.0 ± 56.4	430.0 ± 9.4	0	0
LW10	0	0	177.0 ± 24.5	585.0 ± 98.1	0	0
LW38	0	0	264.0 ± 7.3	76.0 ± 4.9	104.0 ± 4.9	0
ID71	0	0	2744.0 ± 350.0	150.0 ± 85.9	0	0
ID73	0	0	948.0 ± 159.5	486.0 ± 201.1	0	468.0 ± 66.2
LW18	0	0	530.0 ± 9.8	940.0[a]	0	14.0[a]
LW13	0	0	156.0 ± 44.1	286.0 ± 34.3	0	0
LW8	0	0	636.5 ± 136.9	283.1 ± 45.3	0	0
LW14	0	0	389.4 ± 63.4	231.9 ± 21.3	0	66.0 ± 64.1
LW3	0	0	629.0 ± 34.2	0	0	0
Mean ± SD	23.7 ± 91.9	0	561.5 ± 646.9	441.3 ± 361.1	15.5 ± 28.5	103.8 ± 174.8

[a] Single sample tested

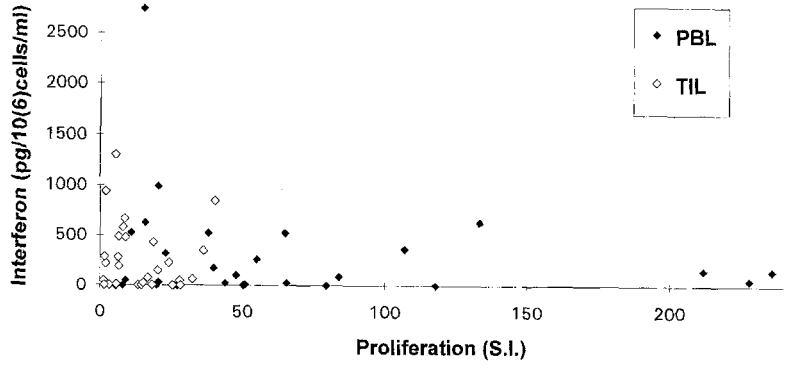

Fig. 2 Relationship between proliferation and interferon γ production. Results are shown for all TIL and PBL cultures stimulated with PMA + IOM or SEB. Regression analysis confirmed there was no correlation between proliferation and interferon γ production for either treatment or for either cell type

Table 2 Tumour necrosis factor α in paired cultures of TIL and PBL stimulated with concanavalin A (*ConA*) or interleukin-2 (*IL-2*)

	Tumour necrosis factor α [pg $(10^6$ cells$)^{-1}$ml^{-1}]					
Experiment	PBL control	TIL control	PBL ConA	TIL ConA	PBL IL-2	TIL IL-2
ID62	14.6 ± 3.07	0	29.9 ± 2.25	0	13.5 ± 0.89	0
ID58	0	0	11.3 ± 12.24	0	0	0
LW31	0	0	49.2 ± 38.26	49.2 ± 102.2	9.5 ± 14.52	99.9 ± 17.67
ID47	142.3 ± 15.98	0	220.5 ± 20.82	0	553.3 ± 263.9	0
LW3	0.5	6.0 ± 33.41	276.6 ± 173.8	127.9 ± 44.60	0	43.0 ± 1.07
ID61	0	0	5.8 ± 20.51	0	0	0
Mean ± SD	26.2 ± 57.2	1.0 ± 2.5	98.9 ± 118.2	29.5 ± 52.1	96.1 ± 224.1	23.8 ± 41.1

Values are means ± SD of duplicate samples

supernatants from the ConA- and IL-2-treated cultures were used to measure TNFα concentration (our experience was that IFNγ levels would be below the detection limits of the immunoassay in these cultures). TIL produced significantly less TNFα than did PBL in ConA-stimulated cultures ($P < 0.05$), but not in control or IL-2-stimulated cultures (Table 2). There was no association of proliferation, or IFNγ and

TNFα production with disease type or stage (data not shown).

Discussion

There is an increasing body of evidence indicating that human tumours elicit immunological responses in situ. In a number of tumours, infiltrating T cells have been shown to use restricted Vβ segments in their T cell antigen receptor suggesting antigen-specific selection within the tumour [2, 21, 26]. TIL expanded in vitro with IL-2 have been found to secrete cytokines in response to autologous tumour cells in colorectal carcinoma [8], melanoma [14], and breast carcinoma [4]. In tumour sections, TIL, including those in colorectal cancer, may express markers of lymphocyte activation e.g. IL-2 receptor, CD45R0, HLA-DR [6, 22]. In some cases a correlation of mononuclear cell infiltration with improved prognosis has been shown but this is by no means a general finding. For example, in colorectal cancer Svennig et al. [28] and Werkmeiser et al. [31] reported a positive correlation, while Koch et al. [13] and Skinner et al. [27] found no relationship.

The failure of tumour-specific responses to become sustained and effective may arise by a number of mechanisms. Possibly some or all of these are necessary steps in the malignant process:

1. Selective loss of HLA class I or II alleles [10, 16] or defects in antigen processing [11] preventing effective antigen presentation.
2. Lack of accessory molecules required for lymphocyte activation, which may lead to anergy.
3. The tumour may produce immunosuppressive agents, for example prostaglandins or TGFβ [23, 29]. The release of tumour antigen may also be immunosuppressive. In colorectal cancer soluble carcinoembryonic antigen has been reported to inhibit lymphokine-activated killer cell killing [12].

In this study TIL from a series of colorectal carcinomas were found to be relatively anergic in that proliferative responses in vitro were very significantly poorer than those of PBL from the same patient. This was not due to paucity of T cells or $CD4^+$ cells in the TIL preparation. The panel of stimulators was selected to provide a range of activation mechanisms. The lectin ConA activates both B and T lymphocytes by cross-linking membrane molecules. In T cells it is thought to act predominantly through CD3 since Jukatt cells lacking CD3 do not respond. A low dose (25 U/ml) of IL-2 will predominantly stimulate previously activated cells already expressing high-affinity IL-2 receptors (approximately 4% in normal PBL). Some studies have reported this response to be higher in TIL than PBL [25]. However, in our study PBL produced more IL-2 than did TIL with all stimulators, and unstimulated TIL did not spontaneously secrete significant amounts of IL-2 despite the observation by ourselves and others that they often express markers of activation. SEB, in common with other superantigens, directly binds HLA-DR and the Vβ region of the T cell receptor in an area outside the peptide groove. PMA + IOM directly activate protein kinase C and mobilise intracellular calcium respectively, bypassing the requirement for CD3 ligation and the resulting series of phosphorylations leading to protein kinase C activation.

We found the proliferation of TIL to be suppressed (2.2–5.0 times compared to PBL) for all treatments, suggesting that this impairment is not restricted to the early stages of lymphocyte activation. However the TIL were able to produce IFNγ in amounts similar to PBL when stimulated with either SEB or PMA + IOM, indicating that the cells have the capacity, given effective stimulation, to produce this cytokine. This was true in a preliminary study of three TIL/PBL pairs for the whole panel of stimulators, in the remainder only PMA + IOM and SEB were used. Cultures with ConA and IL-2 were excluded because IFNγ concentrations were found to be at or below the detection level of the assay and therefore useful comparisons could not be made. However TNFα was found to be produced in control, and ConA or IL-2-stimulated cultures from the 6 patients tested. In this case TIL produced significantly less TNFα than did PBL in the cultures with ConA ($P < 0.05$), but similar amounts in the unstimulated or IL-2-stimulated cultures. Obviously this study would need to be extended beyond 6 patients to verify this result. However, it does illustrate that the expression of different cytokine genes is separately controlled even in situations where lymphocyte proliferation is suppressed.

Others have reported TIL from a variety of tumours to show poor proliferative responses in vitro [15, 18, 25]. Reports of TIL cytokine expression by various methods are less consistent. Rubbert et al. [25] assayed cytokine production by in vitro stimulated TIL from breast cancer and found IFNγ, TNFα but not IL-2 secretion to be impaired compared to PBL. In another study IFNγ and TNFα levels were measured in soluble extracts from fresh colorectal cancer or normal gut and found to be higher in the tumour [19]. IFNγ was produced by CD4 cells and decreased with advancing disease, suggesting a possible antitumour role. TNFα was produced by macrophages and increased with tumour burden. Luscher et al. [14] looked at cytokine gene expression in ten malignant melanomas, which had infiltrates of apparently activated lymphocytes, but found low production of IFNγ, IL-4 and IL-2. As the TIL could be activated to secrete cytokines in vitro by autologous tumour cells the authors suggested that TIL within the tumour were reversibly anergised, possibly because of a lack of effective costimulatory signals in vivo. In renal cell carcinoma, Alexander et al. [1] found that while TIL proliferation

was poor compared to PBL, TIL nonetheless produced IL-2 and expressed IL-2 receptors comparably with PBL. These authors proposed that there may be selective loss in TIL of an intracellular signalling pathway required for proliferation but not for cytokine production.

Although TIL in our study were able to produce IFNγ in vitro we cannot be sure that they do so in vivo because of the unknown antigen stimulation and possible antagonistic factors within the tumour. We have found in an overlapping series of colorectal cancer patients that frozen sections from 50/50 tumours were negative for HLA-DR or ICAM-1 despite the presence of activated inflammatory cells in all tumours and heavy infiltration in 6. Interestingly only 8/40 (20%) of fresh primary colorectal tumour cultures were induced by 100 U/ml IFNγ to express ICAM-1 and HLA-DR [5]. The observation that the tumour cells themselves are apparently resistant to cytokine-mediated induction of immune accessory molecules would clearly restrict the development of an effective immune response, despite the potential of TIL to produce IFNγ.

This study confirms previous reports showing that TIL have a deficient proliferative potential in vitro. Here we have also shown, by using a panel of reagents to drive proliferation, that this apparent anergy is independent of stimulation via the T cell antigen receptor. Interestingly the impaired uptake of tritiated thymidine by TIL (compared to PBL) was not reflected by low IFNγ secretion. Indeed we found no correlation between proliferation and IFNγ secretion, and PBL and TIL produced similar amounts of IFNγ. What accounts for the low proliferation of TIL in vitro remains unclear. It may result from inhibitory factors secreted by tumour cells themselves, or from inefficient antigen presentation within the tumour leading to partial anergy in infiltrating cells. Despite the observation that TIL exhibit reduced proliferative capacity in vitro, if they can produce significant levels of cytokines in vivo, this may be sufficient to develop effective immunity. Further understanding of this process will aid development of future immunotherapeutic strategies.

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