

## CORRELATION BETWEEN EXPRESSION OF CLASS I ANTIGEN AND DEVELOPMENT OF TUMOUR INFILTRATING LYMPHOCYTES (TILS) FROM TUMOUR BIOPSIES OF PATIENTS WITH BLADDER CANCER

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### ABSTRACT

This report presents the findings of TIL development from tumour biopsies of patients with bladder cancer. Analysis of tissue sections showed the presence of T cells in intra-epithelial layer in 8 of 12 cases investigated. In a larger group of patients, TILs were established from 7 of 20 cases and expansion of these cells in Interleukin-2 (IL-2) in the presence of conditioned medium (CM) resulted in increase cell number to as many as 100 fold. Cell surface antigen profile of the cells showed characteristics of activated T cells and showed cytotoxic activity against tumour cell lines K256 and Daudi. In one case where TILs and tumour cell line from the same individual was established, it was found that TILs showed low levels of cytotoxicity against the autologous tumour cells. Investigation of correlation between class I antigen expression and TIL development revealed that all the 7 cases of positive TILs were established from tissue expressing normal levels of free chain of class I antigens. These results are indicative of the presence of IL-2-expandable T cells in tumour biopsy of patients with bladder cancer and demonstrate that the successful expansion of these cells correlates with the normal expression of class I antigens.

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### INTRODUCTION

Recent reports demonstrating that treatment of patients with neoplastic diseases with IL-2 result in durable complete remission has restimulated interest in the immunological mechanism(s) involved in tumour rejection.<sup>1</sup> This has led to the suggestion that the therapeutic efficacy of IL-2 might be due to the clonal expansion of T cells activated by tumour specific antigen(s). The most significant report supporting such a hypothesis has been the findings of Itoh, et al<sup>2</sup> who

showed that IL-2-expandable TILs can be developed from tumours of patients with melanoma. Furthermore, in more than 50% of these cases, TILs showed MHC-restricted killing against the autologous tumour target with little or no killing of allogeneic tumours. In addition, Topalian, et al. demonstrated that compared with autologous IL-2-activated peripheral lymphocytes, TILs were 50 to 100 times more active in tumour killing.<sup>3</sup>

These observations militate against the escape of tumour cells from immunological controls. One possible explanation for the inability of TILs to inhibit tumour progression may in part be due to the abnormal expression of MHC class I antigens by tumour cells.

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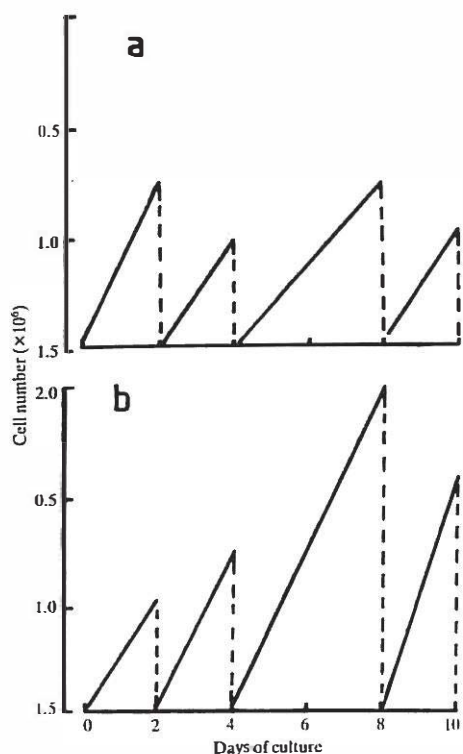


Fig.1. Increase in TIL number in response to IL-2 and CM for FB (a) and FS (b) with increase in the time of culture.

The first clear indication for the loss of these antigens in malignancy was reported by Hui et al.<sup>4</sup> Using animal model, these authors demonstrated that the transfection of missing class I gene into tumour cells lacking these antigens led to a reversal of their tumourigenicity. Human aberrant expression of these antigens have been reported in a number of neoplastic diseases.<sup>5-7</sup> In our own studies<sup>8</sup> investigating bladder tumours, 14 of 18 cases showed some degree of abnormality and this seemed to be correlated with the invasiveness of the tumor.

These observations prompted us to investigate whether TILs can be established from bladder tumour and furthermore, whether there is any correlation between TIL growth and the expression of class I antigens.

### MATERIALS AND METHODS

Operative specimens from the Urology Department of the Royal London Hospital were used immediately after operation. The tissues were divided into two portions the smaller of which was snap frozen and kept in liquid nitrogen for tissue sectioning. The second portion was washed, minced and the resulting cell suspension and tissue fragments were used for developing TILs and tumour cell lines.

The cell preparation was incubated overnight in

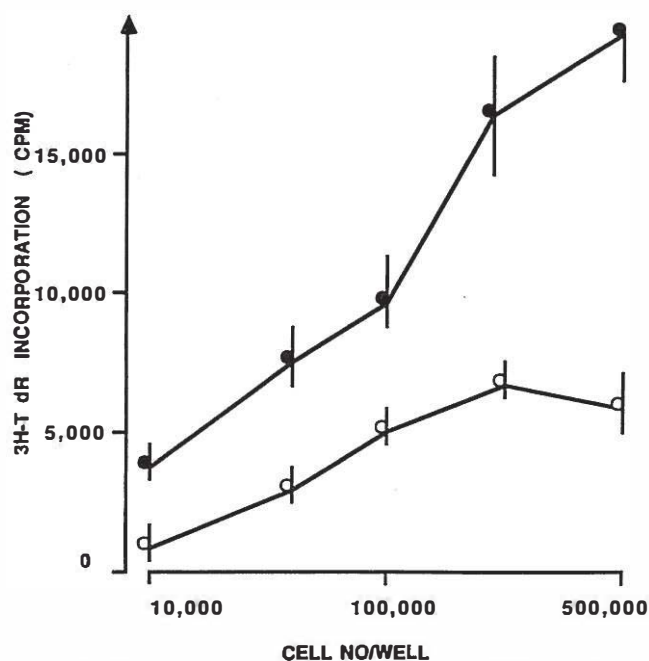


Fig.2. Proliferative response of TILs from FS to IL-2 (100 u/ml) alone (○—○) and IL-2 plus CM (5%, ●—●) for different cell numbers per well.

RPMI containing 10% fetal calf serum in the presence or absence of recombinant IL-2 (100 u/ml, Biogen). After the incubation the non-adherent cells were removed, spun down and resuspended in fresh IL-2 and cultured in a separate flask. In some cases the cell suspension which at this stage contained a large amount of cell debris, was layered onto density gradient solution and after centrifugation, interface cells, ie; mainly lymphocytes were removed and cultured in medium with IL-2. In the cases where successful TILs were established, cells were kept in IL-2 medium and feeding continued every two or three days by adjusting the cell number to  $0.5 \times 10^6$ /ml. After two weeks of culture and the initial expansion of IL-2-receptor bearing T cells, conditioned-medium (CM, 5% v/v, see below) was added in order to increase the rate of cell proliferation. The adherent cells were fed until confluence and were expanded by trypsinising the cells and sub-culturing into new flasks.

Table I. Cell surface phenotype of TILs from different individuals

	w6/32	HB55	CD4	CD8	CD3	NT
FS	98	95	45	50	87	-ve
WIL	98	98	1-2	33	80	-ve
JF	95	83	23	40	89	-ve
FB	100	70	4	30	87	-ve
AW	89	86	27	36	83	-ve
LR	100	93	24	29	69	-ve

Phenotypic characteristics of TILs from six different individuals. Cells were analysed after being in culture for more than 30 days.

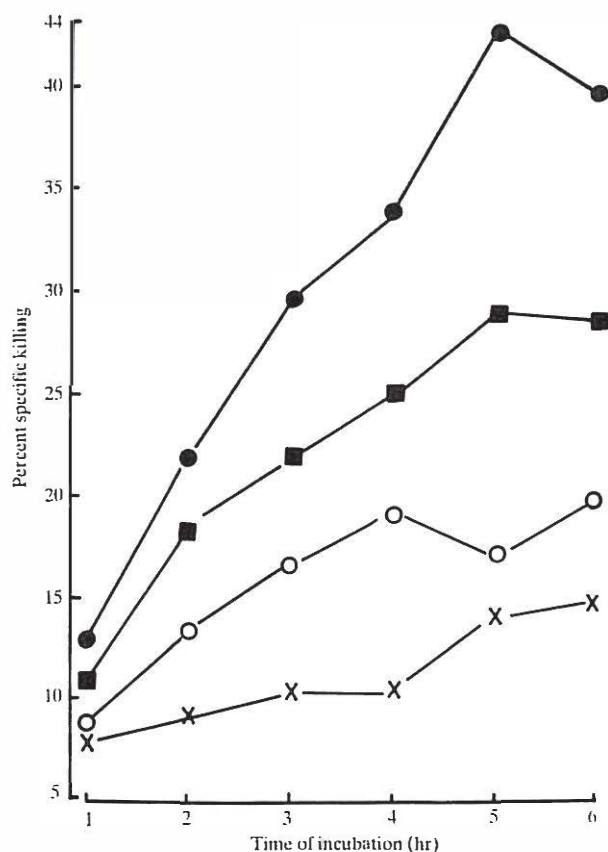


Fig.3. Time course of target (Daudi) killing by effector cells (FS) at E/T ratios of 25/1 (●—●), 12.5/1 (■—■), 6.5/1 (○—○) and 3.2/1 (×—×).

**Conditioned Medium**

This was prepared by activating normal peripheral blood mononuclear cells ( $2 \times 10^6$ /ml) with PHA (2 ug/ml) for two hours at 37°C. The cells were washed three times and culturing continued for a further 36 hrs at above cell number. After the incubation the cell-free supernatant was removed, aliquoted and frozen until use.

**Cytotoxicity**

Established human tumour cell lines Daudi (EBV-transformed B cell line), Molt 4 (T cell line), U937 (monocyticlike cells) and K562(myelocytic cell) or our own established bladder line were labelled with  $^{51}\text{Cr}$

Table II. TIL (WIL) phenotype after different times of culture

Date	12/11/88	1/12/88	22/12/88
CD3	76%	98%	80%
CD4	35%	75%	1-2%
CD8	38%	30%	33%
W6/32	93%	ND	98%
HB55	70%	100%	98%
control	-ve	-ve	-ve

percent positive cells, -ve denotes negative staining.

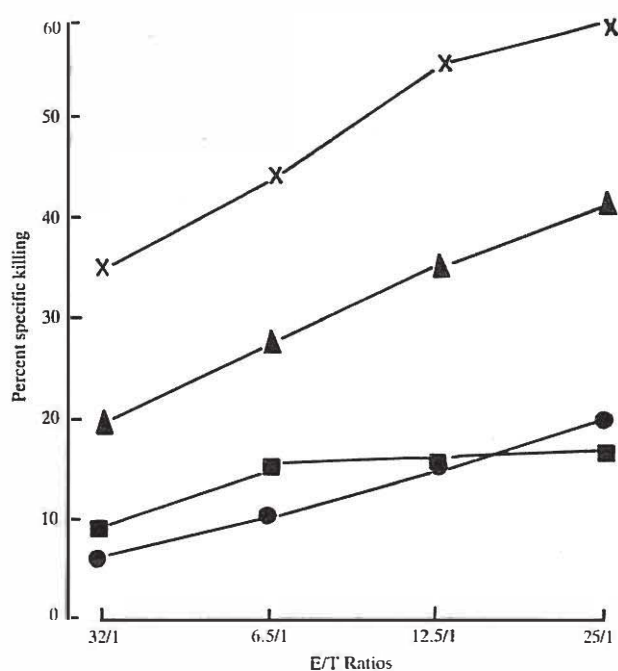


Fig.4. Results are expressed in percent specific killing of Daudi (×—×), Molt 4 (▲—▲), U937 (■—■) and K562 (●—●) by TILs (WIL) at different effector/target ratios.

(250 uCi/target) for 1 hr at 37°C followed by extensive washes. These cells i.e., target cells (T), were mixed (three replicates/treatment) with TILs i.e. effector cells (E) to give E/T ratios of 3.2/1, 6.5/1 and 25/1 in round-bottomed microtitre plates. The cell mixtures were incubated for different length of times after which cell-free supernatants were removed and counted using a gamma counter. The specific killing activity for each treatment was calculated using standard formulae.

**Fluorescent Staining**

Cell suspension was prepared in round-bottomed tube to give  $0.5 \times 10^6$ /tube. After centrifugation, supernatant was discarded and cells were resuspended in 50 ul of appropriate antibody and incubated for 45 mins at room temperature. Cells were washed in PBS and FITC-conjugated rabbit anti-mouse i.e. 2nd antibody (1/50 dilution, Dakopatts) was added and incubation continued for a further 45 mins. After three washes, the cell pellets were used for FACS analysis.

**Tissue Staining**

Frozen sections were cut using a cryostat at a thickness of 5 um, placed on microscope slides, air dried and at -40° until use. A peroxidase-antiperoxidase staining method was employed as previously reported by Nouri, et.at.<sup>8</sup>

**Monoclonal antibodies**

The monoclonal antibodies (Mabs) used as primary



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reagents in the form of tissue culture supernatants, are listed as follows together with their specificities: W6/32 detects all  $\beta$  2 m-associated HLA-A,B,C antigens<sup>9</sup> HC10 detects non- $\beta$  2 associated HLA-A,B,C antigens,<sup>10</sup> L243 detects HLA-DR antigen,<sup>11</sup> anti-CD3, -CD4 and /CD8 (Ortho-pharmaceutical) detect total T, T helper and T suppressor/cytotoxic lymphocytes subsets respectively.

### Cell Proliferation

Proliferation of cells was measured by incorporation of tritiated thymidine (<sup>3</sup>H-Tdr, 0.1 uCi/well, Amersham) into cells. TILs were dispensed into round-bottomed microtitre plates at  $0.5 \times 10^6$ /well in three replicates and incubated in the presence or the absence of stimulus for 48 hr, the last 4 hr of which was in the presence of <sup>3</sup>H-Tdr. The degree of <sup>3</sup>H-Tdr uptake by the cells was measured by harvesting the cells onto filter paper and counting radioactivity in a scintillation counter.

## RESULTS

### Primary Cell Culture

In total, 20 cases with predetermined stage of tumour invasiveness were chosen for establishing TILs of which 7 showed positive growth. The pattern of cell proliferation of two such TILs (FB and FS) over a period of 10 days are presented in Fig. 1. As can be seen, the cell numbers increased at least twofold every 48 hrs and in the case of one individual (FS), this reached 37.2 folds over a period of 10 days. Analysis of TIL proliferation of a representative TILs (FS) to IL-2 alone and IL-2 plus CM is shown in Fig. 2. As can be seen, the cell number of IL-2-activated cells increased further in the presence of CM. Thus at  $0.5 \times 10^6$  cell/well, the <sup>3</sup>H-Tdr uptake of IL-2-activated cells increased from 5,200 cpm to 19,800 cpm indicating the additive effect of CM. This increase was not due to the carried over PHA which might have been present in the CM, since a 0.1 ug/ml equivalent to 5%, PHA had no stimulatory activity on fresh lymphocytes (data not shown).

### Cytotoxicity

Cytotoxic activity of TILs from an individuals (FS) against Daudi cells after different times of incubation was investigated, the results of which are presented in Fig. 3. As can be seen, at all the E/T ratios the longer the incubation period the greater the degree of tumour killing. Furthermore, as the ratios of E/T increased the degree of cell killing also increased. Thus at 4 hrs the specific killing at 3.2/1 and 25/1 ratios were 10.2, 19.625 and 44% respectively and the 4 hr incubation was chosen for subsequent experiments. The standard deviation between the replicates of these and subse-

quent experiments were all below 10%.

The ability of cultured TILs (expanded in vitro for more than two weeks) to kill different well established allogeneic human cell lines was investigated, the results of one individual (WIL) TIL are presented in Fig. 4. As expected there was a direct correlation between E/T ratios and tumour killing. Daudi cells were found to be the most sensitive targets followed by Molt 4 and U937 and K562 the least sensitive. Thus, the percent killing for Daudi cells at 25/1, 12.5/1 and 3.2/1 were 62, 59, 44 and 35% respectively. The degree of cytotoxicity against Daudi cells by TILs from the other 6 individuals at 25/1 E/T ratio varied from 26% to 62%. In addition, TILs from WIL (same individual from which permanent cell line was established) were found to be capable of killing autologous tumour cells by 15% at 25/1 E/T ratio compared with 7% against epithelial cell line SKV14 (tumour cell of similar origin) indicating the low level of cytotoxicity of the TILs against autologous tumour cells.

### TIL phenotypes

The results of cell surface phenotyping for different TILs after being in culture for more than 30 days were assessed, the results of which are presented in Table I. As can be seen, the percentage of CD3, class I and II positive cells was greater than 69% for all the six cases. The percent CD8 positive cells was between 29% to 50%, whereas CD4 positive cells showed greater variability ranging from 1-2% (WIL) to 45% (FS). Furthermore, in all the cases, the percent CD4 positive cells was lower than CD8 positive cells. TILs from one individual (WIL) frozen at different times of culture was analysed for cell surface markers, results of which are presented in Table II. As can be seen, the percent CD3 and CD8 positive cells remained relatively constant throughout the culture period, whereas CD4 positive cells showed an initial increase followed by decrease. Thus the percentage of CD4 positive cells at

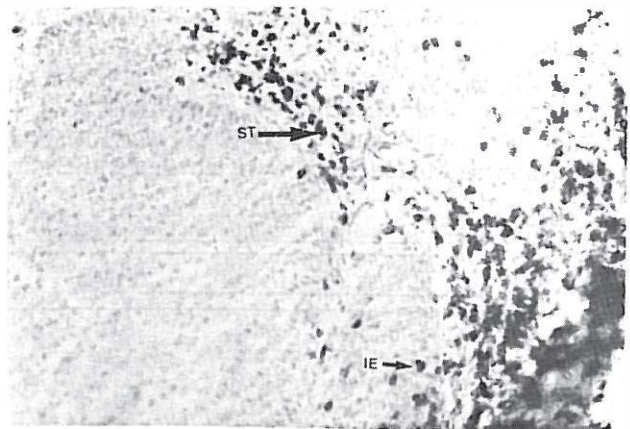


Fig. 5 CD3 positive cells are demonstrated here in bladder tissue section of FS. Positive cells are present in both tumour stroma (st) and in the intra-epithelial (ie) areas.

12/11/88, 1/12/88 and 22/12/88 were 35, 37 and 1-2% respectively. The percentage of class I and II positive cells in the same culture period remained above 70%, indicating either the preferential expansion of CD8 cells or depletion of CD4 positive cells. The analysis of CD3 positive cells in 12 tumour biopsies showed the presence of both CD4 and CD8 cells in tumour stroma (11 cases) and within the tumour epithelial (8 cases) and example of which is shown in Fig.5 (FS).

#### TIL Development and Class I Antigen Expression

Analysis of class I antigen on tumour biopsies using HC10 Mab showed comparable intensity of antigen expression between tumour and tumour stroma in 14 of 20 and in the remaining 6 cases the tumour showed weak or negative staining. The number of positive TIL growth among the above two groups were 7 of 14 and 0 of 6 respectively, indicating that TILs can be more successfully developed from tumours expressing normal levels of class I free heavy chain.

### DISCUSSION

The results of this investigation have revealed that (a) there are activated T lymphocytes in tumour biopsies of some bladder cancer patients and they can be expanded in vitro in response to IL-2 to as many as hundred folds; (b) they express phenotypes of normal activated T cells; (c) they are capable of lysing established allogeneic tumour cell lines; (d) TILs from one individual from whom tumour cell line was established, showed low levels of cytotoxicity against the autologous tumour cells; (e) there seem to be a correlation between the expression of free chain of class I antigens on tumour and the success of TIL growth.

It is well established that IL-2 acts on its receptor expressed on activated T cells, resulting in their clonal expansion.<sup>12</sup> The findings of this study implies that there are activated T cells in bladder tumours of some of these patients capable of proliferating in response to IL-2. This conclusion was reinforced by the observation that there are T cells present at the tumour site in 8 of 12 cases investigated. The most convincing evidence demonstrating the capacity of TILs to kill autologous tumour cells in a MHC-restricted fashion, has been reported in melanoma.<sup>2</sup> Taken this and the demonstration that lymphocyte infiltration into tumour is a good prognostic factor for tumour regression,<sup>13,14</sup> rises the question why bladder tumours escape cytolytic effect of the cytotoxic T cells. One possible explanation for this might be the frequent abnormal expression of class I antigens in these tumours. This is based on our earlier study demonstrating that 14 of 18 cases investigated showed some degree of abnormality in expression of

class I antigens.<sup>8</sup> It is tempting to argue that the failure of clinical efficacy of IL-2 in these patients may in part be due to this abnormality.

The low level cytotoxicity of WIL TILs against autologous tumour cell line is in agreement with the above conclusion, since analysis of MHC class I antigens on these tumour cells demonstrated the absence of HLA-B locus antigens (B7 and B44).<sup>15</sup>

Hence the loss of these antigens may have rendered tumour cells resistant to cytolytic activity of cytotoxic T cells. The finding that TILs were more successfully developed from tumours expressing normal levels of class I antigens seems to be a novel observation. Although, the mechanism(s) for this is not clear, it is possible to envisage that T cells infiltrate and possibly proliferate at the tumour site where there is normal expression of class I antigens acting as an associative molecules for the presentation of putative neo-antigen(s). This possibility is currently under investigation.

In conclusion, the findings of these studies have demonstrated that TILs can be developed from about 30% of bladder tumours in sufficient quantities for immunological analysis. It is envisaged that such a approach may provide opportunities for re-introducing the missing class I antigens by transfecting appropriate genes into autologous tumour cells and investigating the nature of putative neo-antigen(s) which may be present on the tumour cell and these studies are currently in progress.

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