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Effects of Different Culture Protocols on the Expression of Discrete T-cell Receptor Variable Regions in Human Tumour Infiltrating Lymphocytes

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Therapeutic effects of tumour infiltrating lymphocytes (TIL) rely on T-cell receptor (TCR) engagement. In this work, the expression of five TCR α/β variable (V) domains was quantitatively analysed by means of a panel of monoclonal antibodies (Mab) recognising gene products from TCR Vα2, Vβ5, Vβ6, Vβ8 and Vβ12 families in freshly isolated TIL and in autologous peripheral blood mononuclear cells (PBMC) from patients with neoplasms. In 3 out of 6 cases, differences in the expression of Vβ5, Vβ6, Vβ8 or Vβ12 could be detected. TIL populations were expanded by using recombinant human interleukin-2 (rhIL-2) alone or in addition to solid phase bound anti-CD3 Mab. Cultured TIL showed similar CD4/CD8 ratios and cytotoxic activity against autologous neoplastic target cells, regardless of the activation protocol. In 4 patients, the expression of TCR α/β V gene products, as compared with TIL from freshly excised tumours, was found to be modified in cultured TIL, especially in cell populations activated with rhIL-2 only. These results indicate that TCR V gene usage in TIL may quantitatively differ from that in PBMC. TIL culture protocols using rhIL-2 alone or in combination with solid phase bound anti-CD3 may result in differential expression of discrete TCR V families.
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INTRODUCTION

MOST HUMAN neoplasms show some degree of lymphoid infiltration [1], and tumour infiltrating lymphocytes (TIL) from a significant majority of patients can be induced to proliferate to cell numbers compatible with use in adoptive therapies [2–4].

Anti-tumour effects of TIL seem to rely on T-cell receptor (TCR) engagement [5]. Recently, the availability of monoclonal antibody (Mab) and molecular probes identifying specific TCR variable domain (V) gene products has allowed the study of the TCR repertoire involved in the response to a variety of antigens *in vitro* and *in vivo* [6–9].

Use of TIL in adoptive immunotherapies requires *in vitro* expansion by different culture protocols. Therefore, we investigated the effects of these activation steps on the expression of TCR V gene products, as originally represented in TIL at the time of surgical excision.

Thus, a panel of Mab recognising specific TCR V domains, was used to evaluate the quantitative expression of five TCR V gene products in TIL from freshly excised tumours in comparison with autologous peripheral blood mononuclear cells (PBMC). Subsequently, the effects of long-term culture according to two different protocols, on the expression of the TCR V gene products under study were comparatively analysed. TIL were cultured in the presence of recombinant interleukin 2(rhIL-2) alone or in combination with solid phase bound anti-CD3.

Our results suggest that in some patients expression of discrete TCR V genes in TIL is different from that detectable in simultaneously sampled PBMC. Most importantly, we here report that the two protocols most commonly used to expand TIL [2, 3], have different effects on the expression of these TCR V families, as compared with uncultured TIL populations.

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PATIENTS AND METHODS

Patients

7 patients bearing primary or metastatic renal cell carcinoma (RCC, $n = 2$), melanoma ($n = 3$) or colorectal carcinoma ($n = 2$) presenting to our clinic for surgical treatment were studied (Table 1). None of the patients underwent chemo-, radio- or immunotherapeutic treatment before surgery.

Cell preparation and culture

Freshly excised biopsy specimens were mechanically dissociated and single cell suspensions were obtained by enzymatic digestion [2, 3]. The enzyme cocktail used throughout this study contained collagenase type IV, 0.1% w/v, 2.4 U/ml hyaluronidase type V and 0.01% w/v DNAse (all from Sigma Chemical Co., St. Louis, Missouri, U.S.A.) in RPMI 1640 (Gibco, Paisley, Scotland, U.K.) Cells were digested for 3 h, washed and used for cultures or phenotypes. Cell viability after digestion always exceeded 90%, and preliminary tests indicated that enzyme treatment did not affect the expression of the epitopes recognised by the Mab used in this study (data not shown). Autologous venous samples were also obtained at the time of surgery and mononuclear cells were isolated by gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway) by standard techniques. Tumour-derived cell suspensions were resuspended in RPMI 1640 supplemented with glutamine (1 mmol/l), penicillin (50 IU/ml), streptomycin (50 μg/ml) (Gibco) and 5% v/v human AB serum (hereafter referred to as complete medium) at a final

Table 1. Patients' characteristics

Case	Age (years)	Sex	Histology	Tumour site	TIL per gram tumour biopsy	TIL as % of live cells in the single-cell suspension*
1	62	F	Renal cell carcinoma	Primary	5.5×10^6	35
2	53	M	Renal cell carcinoma	Primary	3.5×10^5	11
3	67	M	Melanoma	Lymph node	5.1×10^7	55
4	52	M	Melanoma	Brain	3.3×10^5	2
5	50	F	Melanoma	Lymph node	8.4×10^6	16
6	67	F	Colorectal carcinoma	Lymph node	1.1×10^8	81
7	52	M	Colorectal carcinoma	Lymph node	3.9×10^6	34

*Tumour infiltrating lymphocytes (TIL) were defined as CD45 "bright"/CD14- cells presenting typical forward and side scatter lymphocyte cytometric parameters.

concentration of 2–3 $\times$ 10^5 cells/ml. Cells were subsequently incubated overnight in the presence of rhIL-2, 1000 U/ml (F. Hoffmann-La Roche Ltd., Basel, Switzerland). On the following day half of the cultures were transferred for 48 h to 6-well trays (Costar, Cambridge, Massachusetts, U.S.A.) previously coated with a mitogenic anti-CD3 ϵ Mab, in ascitic form, in a 1:10 000 final dilution (CB3G, a kind gift of Prof. F. Malavasi, Turin, Italy) [10]. Cultures were incubated at 37°C in 5% CO_2 humidified atmosphere and periodically refed with fresh complete medium supplemented with rhIL-2 (200 U/ml). Cultures pulsed with anti-CD3 on day 1 were further stimulated with solid phase bound Mab on day 14.

Flow cytometry

Freshly isolated or cultured cell populations were stained with commercial, fluorochrome-labelled Mab recognising CD3, CD4, CD8, CD14, CD16, CD45, CD56, CD69, HLA-DR and TCR α/β (WT31) or γ/δ (11F2) framework determinants (all from Becton Dickinson, Mountain View, California, U.S.A.). Specific fluorescence was analysed on cells gated according to side scatter and forward scatter cytometric parameters by FACScan, taking advantage of Paint-a-gate software (both from Becton Dickinson). Data were expressed as percentages of total lymphocytes in the gate (CD45 "bright"/CD14-cells). Data concerning CD69, HLA-DR, TCR α/β and γ/δ were obtained in double labelling assays together with anti-CD3 and were referred to as percentages of CD3+ cells.

The expression of TCR α/β V gene products was studied by means of specific Mab in indirect immunofluorescence tests. Seven commercially available Mab were used (Diversi-T panel from T Cell Sciences, Cambridge, Massachusetts, U.S.A.). Three of these recognise partially overlapping Vβ5 subfamilies (clones 1C1, Vβ5a, W112, Vβ5b, and LC4, Vβ5c) [11–13]. Three recognise Vβ6, Vβ8 and Vβ12 gene products (clones OT145, 16G8 and S511, respectively) [14, 15] and Mab F1 [16] identifies a Vα2 subfamily. Another Mab, previously characterised, directed against Vβ8 (17-34-11, Vβ8b), was also used [17]. After staining with specific Mab (30 min at 4°C) cells were washed and incubated in the presence of fluorescein isothiocyanate (FITC) $(Fab)_2$ goat anti mouse Ig antibodies (Jackson Immuno Research, West Grove, Pennsylvania, U.S.A.) for 30 min at 4°C, and washed. Specific fluorescence was evaluated by acquiring 30 000 gated cells per measurement. Data concerning Vα and Vβ were expressed as percentages of α/β+ cells.

Phenotypic analysis of T-cell receptor V domain expression proved to be remarkably reliable in that multiple measurements performed in identical cell populations with the same reagents provided consistently reproducible data, with mean standard deviations never exceeding 2% (data not shown). Based on this, a cut-off value of 4% was selected, and expression of discrete TCR V products was considered as discordant when percent of positive cells in paired cell populations differed by 4% or more.

Cytotoxicity

Cytotoxic activity against natural killer (NK) sensitive K562, NK resistant 1301 cell lines and autologous fresh frozen tumour cells was evaluated by ^{51}Cr release assays as previously described [18]. Briefly, effector cells were washed and resuspended in RPMI 10% fetal calf serum (FCS). Target cells were labelled for 1 h with 5.55 MBq of a $Na^{51}Cr$ solution (Du Pont, Regensdorf, Switzerland), extensively washed and added to the effector cells at different effector:target ratios. After incubation for 4 h at 37°C in humidified atmosphere, culture supernatants were collected and counted with a γ-counter. Specific cytotoxicity was calculated according to the standard formula [10]. Spontaneous mean ^{51}Cr release ranged between 30 and 40% for autologous targets and did not exceed 10% for established cell lines.

RESULTS

Isolation and characterisation of TIL

Tumour or peripheral blood derived cell suspensions were stained with phycoerythrine (PE) labelled anti-CD14 and FITC labelled anti-CD45 and analysed by FACScan. By analysing CD14−/CD45 "bright" cells, characterised by typical forward and side scatter cytometric parameters, we were able to estimate the percentages of lymphocytes in individual cell suspensions. Percent of lymphocytes ranged between 2 and 81% (median: 34%). Absolute numbers of lymphocytes per gram of biopsy specimen ranged between 3.5 $\times$ 10^5 and 1.1 $\times$ 10^8 (median: 5.5 $\times$ 10^6) (Table 1). Percentages of CD3+, CD4+ and CD8+ lymphocytes in tumour suspensions did not significantly differ from those detected in autologous, simultaneously sampled PBMC, as estimated by the Wilcoxon signed-rank test. On the other hand, the percentage of cells presenting a NK phenotype (CD16 and/or CD56+, CD3−) was found to be significantly higher in PBMC than in autologous TIL suspensions (15 $\pm$ 8% vs. 4 $\pm$ 3%) (Fig. 1a).

Significantly higher percentages of CD3+ cells expressing HLA-DR and CD69, as detectable by two-colour fluorescence, were observed in TIL populations, as compared with autologous PBMC (32 $\pm$ 18% vs. 5 $\pm$ 2% and 60 $\pm$ 30% vs. 4 $\pm$ 2%, respectively) (Fig. 1b).

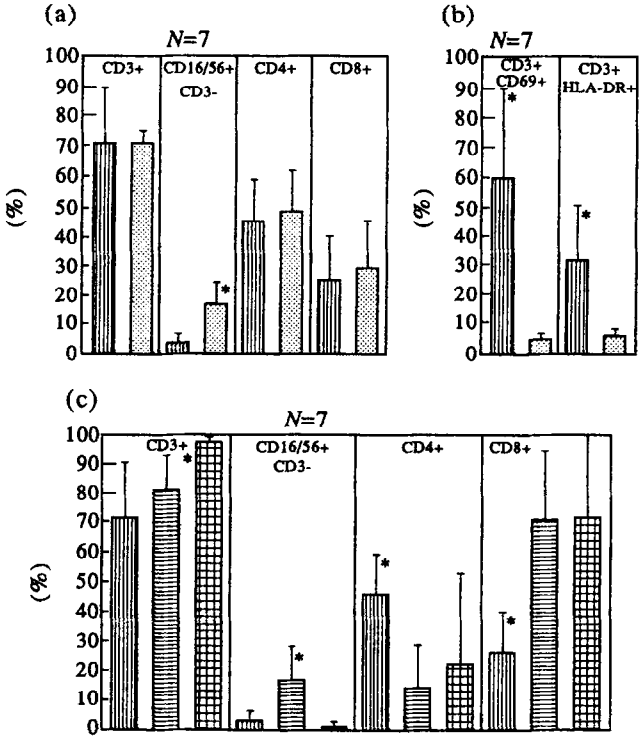

Fig. 1. Phenotypic characteristics of freshly isolated and cultured TIL. (a) TIL from freshly excised biopsy specimens (□) and autologous PBMC (□) were stained with fluorochrome-labelled Mab and analysed by FACS. Percentages of CD3, CD16/56, CD4 and CD8 positive cells are referred to CD45 "bright"/CD14-cell populations characterised by typical lymphocyte forward and side scatter cytometric parameters. (b) expression of activation markers (CD69 and HLA-DR) in TIL (□) and PBMC (□) was evaluated by double labelling with anti-CD3. Data are presented as percentages of CD3+ cells. (c) Data reported concern TIL activated with rhIL-2 alone (□) or rhIL-2 solid phase bound anti-CD3 (□). Data obtained at the time of surgical excision (□) are also shown. All data are reported as percentages of CD45 "bright"/CD14-cells. For any other details see Materials and Methods. Asterisks indicate $P < 0.05$.

TCR analysis in TIL and PBMC at the time of surgery

In 6 patients out of 7 (PBMC from patient 6 could not be obtained in sufficient numbers), we were able to comparatively analyse TCR expression in TIL and autologous PBMC sampled, simultaneously, at the time of surgical excision (Table 2). T-cells expressing TCR α/β were found to be largely prevailing on γ/δ positive T-cells. In 3 patients (2, 3 and 4), however, differences were detected between TIL and PBMC in the percentages of γ/δ cells (1.6 vs. 5.8%, 5 vs. 15% and 10.6 vs. 1.6%, respectively). Regarding expression of TCR V gene products, a major finding was that in 39 out of 44 measurements (88.6%) the percentages of positive cells in paired TIL and PBMC populations consistently differed by 2% or less, and were thus considered "concordant". Differences equal to or greater than 4% could also be reproducibly detected. In RCC patient 2, 13.3% of peripheral blood T-cells expressed a TCR Vβ5 gene product identified by 1C1 Mab, as compared with 4.1% in TIL. In melanoma patient 4, a lower expression of TCR Vβ6 and Vβ8 was observed in TIL from a brain metastasis, as compared with autologous PBMC (4.3 vs. 11.7% and 7 vs. 24.6%). In colorectal carcinoma patient 7 we observed an increased expression of TCR Vβ5, as identified by LC4 Mab and of Vβ12 in TIL as compared with autologous peripheral blood T cells (5.8 vs. 1.8% and 6.1 vs. 2.1%, respectively).

Phenotypic characterisation of cultured TIL

Successful expansion of TIL according to the two protocols could be obtained in all patients except for RCC patient number 2. Cultures of TIL pulsed with anti-CD3 resulted in significantly higher cell proliferation as compared with the "rhIL-2 only" protocol. Indeed, they showed a mean 148-fold expansion, as compared with a mean 19-fold expansion in TIL cultured with rhIL-2 only, after 5 weeks of incubation. Phenotypic characterisation (Fig. 1c) indicated that the great majority of cultured TILs were CD3+, independent of the activation protocol. In TIL activated by rhIL-2 alone, however, the percentage of CD3+ cells was similar to that observed at the time of surgery, whereas a significant increase could be observed in TIL cultured in the presence of anti-CD3 Mab plus rhIL-2. Most CD3+ cells were CD8+ in TIL activated according to either protocol (70 ± 24% for "rhIL-2 alone" vs. 71 ± 31% for "rhIL-2 plus anti-CD3"). A significant increase in the per cent of CD8+ cells, compared with data obtained from biopsies was observed in all patients. No difference was detected in the percentage of CD4+ cells in populations cultured according to either protocol. In all patients, however, the percentage of CD4+ cells was decreased, in comparison with the biopsies, regardless of which activation protocol was used. In contrast, the percentage of TIL displaying CD16+CD56+/CD3− "NK" phenotype was significantly higher in the cell population cultured in the presence of rhIL-2 alone, as compared with the anti-CD3 pulsed TIL (17 ± 11% vs. 1.2 ± 1%) or with uncultured TIL (3.3 ± 3%).

Cytotoxicity assays

TIL populations, activated by the two protocols, were tested in cytotoxicity assays against NK sensitive and NK resistant cell lines and uncultured autologous tumour cells. All tests were performed after 4 weeks of culture. Cytotoxic activity against K562 cell line was found to be significantly higher (54±15% vs. 33±26% at 50:1 E:T ratios, $P = 0.0134$, two tailed paired t-test) in TIL populations cultured in the presence of rhIL-2 only, as compared to those activated according to the "rhIL-2 plus anti-CD3" protocol (Table 3). In contrast, no significant differences could be detected when 1301, a NK resistant cell line, was used as target (median: 13.5%, range: 5–55% and median 12.5%, range 0–34% at 50:1 E:T ratios, for rhIL-2 alone and anti-CD3 plus rhIL-2, respectively). Notably, in patient 4 no cytotoxicity against either K562 or 1301 cell lines could be detected in anti-CD3 activated TIL. Cytotoxic activity against fresh frozen autologous tumour cells was observed in melanoma and RCC patients but not in patients affected by colorectal carcinoma and its extent was not influenced by the activation protocol (median: 6.5%, range: 0–48% and median: 10.5%, range: 0–41% at 50:1 E:T ratios, for rhIL-2 alone and rhIL-2 plus anti-CD3, respectively).

TCR expression in cultured TIL

The great majority of cultured TIL expressed TCR α/β regardless of the activation protocol (Table 4). In patients 1 and 7 percentages of cells expressing TCR γ/δ were significantly higher in "rhIL-2 alone" stimulated T-cells than in unstimulated TIL or in anti-CD3 pulsed TIL, where they did not differ from each other (Table 4). In patient 4 no γ/δ+ cells could be detected in the cultured cell populations, regardless of the activation protocol, as opposed to 10% in freshly isolated TIL. In patient 6, higher percentages of cultured TIL, regardless of the activation protocol, expressed TCR γ/δ as compared with uncultured TIL. A significantly higher percentage, however, could be observed

Table 2. TCR gene product expression in freshly isolated TIL and autologous peripheral blood T-cells

Case*	Cell populations	αβ†	γδ	Vα2a‡	Vβ5a	Vβ5b	Vβb5c	Vβ6a	Vβ8a	Vβ8b	Vβ12a
1	PBMC	95.2	4.8	1.5	1.8	0.2	1.9	4.2	2.3	ND§	0.7
	TIL	95.3	4.7	2.5	2.3	0.9	1.4	2.5	1.5	3.3	1.0
2	PBMC	94.2	5.8‖	6.6	13.3‖	2.5	ND	5.7	6.1	ND	6.8
	TIL	98.4	1.6	6.8	4.1	2.7	ND	4.0	6.0	ND	7.3
3	PBMC	85.0	15.0 ‖	3.7	2.3	0.9	1.8	1.3	4.0	3.3	1.0
	TIL	95.0	5.0	2.9	2.9	1.0	2.6	1.3	4.9	4.9	1.7
4	PBMC	98.4	1.6	6.4	6.4	2.6	5.6	11.7‖	8.7	24.6‖	5.2
	TIL	89.4	10.6‖	4.9	4.6	3.5	5.7	4.3	6.0	7.0	2.5
5	PBMC	98.7	1.3	3.6	2.9	0.9	2.9	3.9	5.4	6.5	1.9
	TIL	97.5	2.5	1.5	1.5	0.8	1.4	2.5	2.8	3.5	1.0
7	PBMC	98.5	1.5	3.7	1.7	1.5	1.8	5.9	3.1	4.5	2.1
	TIL	98.0	2.0	3.7	4.0	3.9	5.8‖	3.0	5.3	ND	6.1‖

*PBMC from patient 6 were not available. †Data are reported as percent of CD3+ cells. ‡Data are reported as percent of TCRαβ cells. §ND = Not done. ‖Differences ≥ 4%.

in TIL cultured in the presence of "rhIL-2 alone" as compared with anti-CD3 pulsed TIL.

Expression of TCR α/β V gene products detectable by our Mab panel was studied in TIL from 6 patients, where we could compare data obtained in cell populations cultured according to the different protocols. Phenotypes were analysed after 4–5 weeks of culture. Representative histograms, referred to patient's 4 TIL, cultured according to either protocol are reported in Fig. 2. In two thirds of the paired measurements (36/48) differences in the per cent of cells bearing V gene products recognised by our Mab panel in the two cell populations never exceeded 2%. In contrast, in patient 4, about 25% of the "rhIL-2 only" activated TIL expressed TCR Vβ chains recognised by 1C1 and W112 anti Vβ5 Mab, as opposed to 3.3% and 1.6%, respectively, in TIL activated with anti-CD3 and rhIL-2. On the other hand, expression of Vβ8 gene products identified by 17-34-11 Mab was increased in anti-CD3 pulsed TIL (Table 3). These differences were repeatedly detected in serial measurements performed at different culture times (data not shown).

Table 3. Cytotoxic activity against different target cells in cultured TIL

Activation protocol	IL-2*			Anti-CD3 and IL-2†		
Target	K562	1301	Autologous tumour cells	K562	1301	Autologous tumour cells
Case‡						
1	72§	55	4	66	34	14
3	41	36	48	18	21	41
4	36	5	9	0	0	7
5	72	17	24	61	17	21
6	48	5	0	36	8	0
7	55	10	0	16	8	0

*TIL were cultured for 4 weeks in the presence of rhIL-2 (200 U/ml).
†TIL were cultured for 4 weeks in the presence of rhIL-2 (200 U/ml) and pulsed with solid bound anti-CD3 Mab on day 1 and 14.
‡"*In vitro*" expansion of TIL from patient 2 could not be obtained.
§Mean percentage of specific ^{51}Cr release at a E:T ratio of 50:1, derived from triplicates tests.

The expression of TCR V gene products was then analysed in activated TIL according to either protocol in comparison with uncultured TIL, as observed on the day of surgical excision. In 10 out of 47 paired measurements (21.2%) the expression of discrete TCR α/β V-gene products was found to be discordant between rhIL-2 activated TIL and their uncultured counterparts. In TIL activated with rhIL-2 plus anti-CD3, such differences were observed in only 3 out of 47 paired measurements (6.4%). In patient 4, the percentage of cells expressing TCR Vβ5 and Vβ8 at the time of surgical excision or after 4 weeks of culture according to the rhIL-2 plus anti-CD3 protocol did not show differences higher than 2%. In contrast, as reported above, about 25% of TIL, activated with rhIL-2 only, expressed Vβ5, but 2.5% only expressed Vβ8. In patient 5 an increase in Vβ8+ cells, as compared with uncultured TIL could be detected exclusively in "rhIL-2 only" activated TIL. In patient 6, 12.7% of freshly isolated TIL expressed Vβ8, as opposed to 2.6% or 3.4%, depending on the antibody used, in TIL activated with rhIL-2 only. The percentage of T cells expressing Vβ8 was also reduced in TIL activated with rhIL-2 plus anti-CD3 (6.2% and 6% with the two Mab). Expression of Vβ12 was also decreased in cultured TIL regardless of the activation protocol, while the percentage of Vα2+ cells was reduced mainly in "rhIL-2 only" cultured TIL. Finally, TCR Vβ5c expression was virtually absent in "rhIL-2 only" cultured TIL from patient 7, as compared with 5.8% and 4.6% in uncultured and in anti-CD3 pulsed TIL, respectively.

DISCUSSION

Tumour infiltration by lymphocytes is often considered to result from an abortive immune response against autologous neoplastic cells [1]. Further, in some neoplasms the extent of infiltration can be correlated with patients' survival [19]. Based on these findings and on the availability of recombinant cytokines, protocols have been designed to expand TIL *in vitro* for adoptive immunotherapy trials [2–4]. In particular, TIL have been stimulated with rhIL-2 alone or in combination with solid phase bound anti-CD3 in order to obtain quantities of cells suitable for therapeutic use [2, 3]. Since the effects of TIL appear to be mediated by TCR engagement [5], the analysis of the TCR V gene usage in TIL at the time of the surgery, and of

Table 4. TCR gene product expression in freshly isolated and cultured TIL

Case*	Cell populations	αβ†	γδ	Vα2a‡	Vβ5a	Vβb5b	Vβ5c	Vβ6a	Vβb8a	Vβ8b	Vβ12a
1	Fresh TIL§	95.3	4.7**	2.5	2.3	0.9	1.4	2.5	1.5	3.3	1.0
	TIL rhIL-2‖	90.6	9.4	2.0	1.9	0.9	1.1	2.3	1.7	2.4	1.0
	TIL rhIL-2 + anti-CD3¶p	96.8	3.2	1.9	4.2	0.7	0.7	1.5	2.3	2.8	0.8
3	Fresh TIL	95.0	5.0	2.9	2.9	1.0	2.6	1.3	4.9	4.9	1.7
	TIL rhIL-2	97.4	2.6	2.6	4.7	1.7	1.4	0.7	5.6	5.7	2.7
	TIL rhIL-2 + anti-CD3	94.8	5.2	3.4	3.4	0.9	1.5	0.8	3.9	4.7	2.1
4	Fresh TIL	89.4	10.6**	4.9	4.6	3.5	5.7	4.3	6.0	7.0**	2.5
	TIL rhIL-2	100.0	0.0	3.9	25.7**	26.0**	4.8	0.9	2.4	2.5	0.8
	TIL rhIL-2 + anti-CD3	100.0	0.0	3.8	3.3	1.6	3.9	4.8	5.8	6.7**	2.4
5	Fresh TIL	97.5	2.5	1.5	1.5	0.8	1.4	2.5	2.8	3.5	1.0
	TIL rhIL-2	95.9	4.1	4.2	2.7	1.0	3.6	2.8	7.6**	7.8**	2.3
	TIL rhIL-2 + anti-CD3	97.9	2.1	3.7	3.1	1.6	0.5	2.5	5.2	4.5	2.3
6	Fresh TIL	97.7	2.3	5.7**	4.5	1.6	2.2	1.6	12.7**	12.7**	6.3**
	TIL rhIL-2	78.5	21.5**	1.5	1.2	0.8	1.4	0.2	2.6	3.4	1.3
	TIL rhIL-2 + anti-CD3	89.9	10.2**	2.9	3.9	1.6	2.9	1.5	6.2	6.0	1.6
7	Fresh TIL	98.0	2.0	3.7	4.0	3.9	5.8**	3.0	5.3	ND††	6.1
	TIL rhIL-2	86.0	14.0**	1.8	1.0	1.0	0.8	1.1	5.0	4.8	4.4
	TIL rhIL-2 + anti-CD3	97.9	2.1	4.8	3.4	1.7	4.6	4.3	6.5	8.0	2.9

**In vitro* expansion of TIL from patient 2 could not be obtained. †Data are reported as per cent of CD3+ cells. ‡Data are reported as per cent of TCRαβ cells. §Percentages are relative to cell suspensions derived from freshly digested tumour biopsy specimens. ‖TIL were cultured for 4–5 weeks in the presence of rhIL-2 (200 U/ml). ¶TIL were cultured for 4–5 weeks in the presence of rhIL-2 (200 U/ml) and pulsed with solid phase bound anti-CD3 Mab on day 1 and 14. **Differences ≥ 4%. ††Not done.

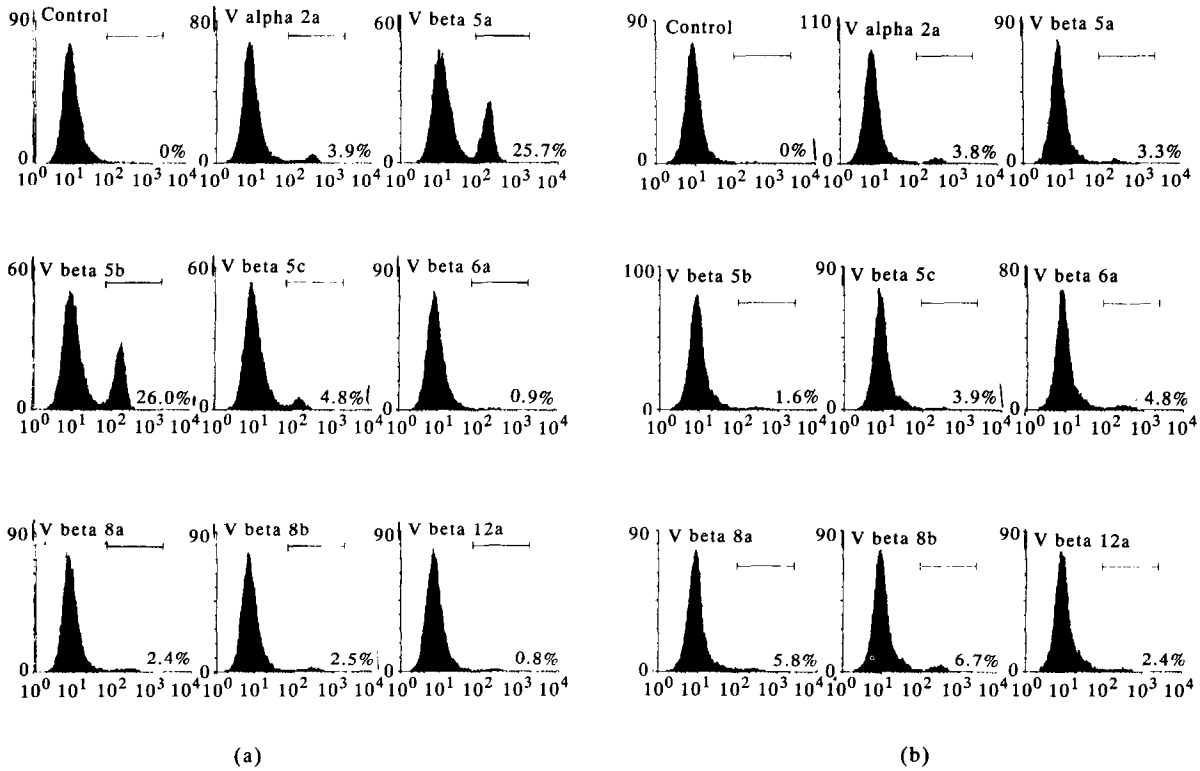

Fig. 2. Expression of specific TCR-V gene products in cultured tumour infiltrating lymphocytes (TIL) from patient 4. TIL were cultured in the presence of rhIL-2 alone (a) or in combination with solid phase bound anti-CD3 (b). Data are reported as percentages of TCR alpha/beta positive T-cells.

its modifications during culture emerge as important issues in the context of cellular adoptive immunotherapies.

Thus, a panel of Mab recognising specific TCR α/β V gene products, and permitting sensitive and reliable quantitation, was used to comparatively evaluate the use of discrete TCR V-gene products in TIL and autologous PBMC. Most importantly, we also analysed modifications induced by the different protocols currently used to expand TIL in culture for clinical adoptive immunotherapies.

In humans, TIL from melanomas have been shown to use a restricted number of Vα gene products [20]. In contrast, quantitative comparison with autologous PBMC has only been reported for ascitic ovary tumours [21] and we are not aware of works addressing the effects of different stimulation protocols on the TCR repertoire of cultured as opposed to "resting" TIL.

Our data show that differences in the per cent of cells expressing Mab defined TCR V domains can be detected between resting, peripheral blood and autologous tumour derived T cells, as sampled at the time of surgical excision, in 3 patients out of 6. The TIL identity of the cell populations studied is validated by the fact that, in contrast with PBMC, T cells derived from tumour biopsy specimens express in high percentages activation markers such as CD69 and HLA-DR. On the other hand, no significant difference in the per cent of CD4+ and CD8+ could be detected between freshly derived TIL and autologous PBMC. It is also of note that in 3 patients differences could be observed in the percentages of T cells bearing γ/δ TCR. These findings are compatible with at least two hypotheses. First, they might be the result of a positive selection mechanism in the TCR repertoire in either cell population, possibly related to an antigenic recognition. Alternatively, they might be a consequence of the selective shut off of T-cells expressing discrete TCR V-gene products [22], as already described with staphylococcal enterotoxin "superantigen" priming in animal models [23] or in AIDS [24]. Both mechanisms might be simultaneously involved.

Data concerning the expression of the TCR V-gene products identified by our Mab in cultured TIL could be of clinical interest. In the TIL population cultured according to the "rhIL-2 plus anti-CD3" protocol, the expression of V gene products detectable by our reagents, resembles the one in uncultured TIL, since differences exceeding 4% are only detectable in three out of 47 paired measurements. This might depend on activation of all T lymphocytes present in the cell suspension upon CD3 triggering, as also suggested by the high rate of cell expansion. In contrast, in "rhIL-2 only" cultured TIL, the original TCR V gene usage appears to be less respected. The mechanism underlying this *in vitro* selection is unclear, but might be related to preferential expansion of *in vivo* preactivated TIL.

Cytotoxic activity against NK sensitive K562 cell lines was found to be higher in "rhIL-2 only" cultured TIL populations, where higher percentages of CD16/CD56+, CD3− cells could be demonstrated. In contrast, we could not find any correlation between cytotoxic activity of cultured TIL against autologous tumour cells, mainly detectable in melanoma patients, and differential TCR V usage neither at the time of surgical excision nor after culture, regardless of the activation protocol. Further, cytotoxic activity against autologous neoplastic cells was comparable in cultures activated in the presence of rhIL-2 only or in combination with anti-CD3, the latter method, however, generating much larger numbers of TIL.

Our results clearly indicate that the two most widely used TIL culture protocols differentially affect TCR V gene usage. Overexpansion of TIL unrelated with specific tumour recognition, or failure to induce efficient proliferation of specific T-cells might influence the clinical outcome of adoptive cell therapies. At the present time, however, no univocal *in vitro* functional test is available, predicting superior *in vivo* efficacy of either protocol [4].

Altogether, these findings address the issue of the relationship between fresh TIL and their cultured counterparts, directly focusing on the molecular structure specifically devoted to the recognition of so far poorly defined tumour antigens. Indeed, they indicate that protocols inducing the expansion of TIL populations characterised by similar CD4/CD8 ratios and comparable cytotoxic activity against autologous tumour cells can result in relevant differences in the TCR repertoire.

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Changes in Glycosylation of L1210 Cells After Exposure to Various Antimetabolites

Truus W. De Graaf, Sabrina S. Slot, Godefridus J. Peters and Willem Van Dijk

This study establishes that antimetabolites do have the potency to change cellular glycosylation, as was suggested in our previous review (*Eur J Cancer* 1990, 26, 516–523). Murine leukaemia L1210 cells were exposed to various antimetabolites under non-lethal conditions. The antimetabolites 5-fluorouracil (5FU), arabinofuranosylcytosine (AraC), methotrexate (MTX) and 6-mercaptopurine (6MP), but not 6-thioguanine, induced considerable changes in the metabolic incorporation of radioactively labelled monosaccharides. Each antimetabolite exhibited a different effect. Significant differences were found between the radioactivity incorporated from the monosaccharides glucosamine, fucose, mannose and galactose, relative to control values. Polyacrylamide gel electrophoresis indicated that changes were induced in the glycosylation of individual glycoproteins. 5FU, AraC, MTX and 6MP all influenced both pyrimidine- and purine-mediated sugar incorporation. This excludes, therefore, direct effects of the antimetabolites on their analogue nucleotide-sugars. The antimetabolite-induced changes in glycosylation did not directly correlate with the observed cell-cycle effects of the antimetabolites.
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INTRODUCTION

ANTIMETABOLITES ARE widely used in the treatment of neoplastic diseases. They are able to interfere with nucleotide metabolism, thus inhibiting RNA/DNA synthesis, which can lead to cell death. Various additional effects, possibly interrelated, have been reported: (i) induction of differentiation [1–3], (ii) blockade in a particular phase of the cell cycle [4–6], (iii) augmentation of the antigenic activity of glycoproteins [7], (iv) change of cellular nucleotide-sugar metabolism and/or glycoconjugate biosynthesis [8].

Changes in the glycosylation of cells occur during normal maturation and differentiation, but also during malignant development [9–11]. The evidence is compelling that cell–cell interactions are affected by changes in cell–surface glycosylation, e.g. during the processes of adhesion, invasion and metastasis [12–15].

Some examples of observed antimetabolite-induced changes in glycosylation [8] are: (i) altered glycosyltransferase activity [16], (ii) changed lectin binding [17], and (iii) altered incorporation of ^{3}H-labelled sugars [2, 3, 18, 19]. In the latter studies it was not excluded, however, that antimetabolite-induced changes in specific radioactivities of the incorporated label might have caused the alterations.

The mechanism of action by which antimetabolites can affect glycosylation is not completely understood [8]. Pyrimidine and purine antimetabolites could interfere with, respectively, the cellular pyrimidine and purine nucleotide-sugar pools (e.g. UDP-glucosamine and GDP-fucose), which are precursors for glycans. Furthermore, antimetabolite-induced changes in the cell-cycle distribution could be responsible for changes in cellular glycosylation [20, 21].

This study has been performed in order to investigate: (i)

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