International Immunology, Vol. 9, No. 8, pp. 1073–1083

TCR analysis reveals significant repertoire selection during *in vitro* lymphocyte culture

Pierre-Yves Dietrich, Paul R. Walker, Valérie Schnuriger, Philippe Saas, Gaëlle Perrin, Maryvonne Guillard[1], Catherine Gaudin[2] and **Anne Caignard[1]**

Division d'Oncologie, Laboratoire d'Immunologie des Tumeurs, Hôpital Universitaire, 1211 Genève 14, Suisse
[1]INSERM CJF 94-11 and [2]Unité INSERM U333, Institut Gustave Roussy, Villejuif, France

Keywords: glioma, renal cell carcinoma, skewing, TCR β chain, tumor immunology

Abstract

The *in vitro* stimulation of T lymphocytes is frequently used as a technique to expand specific cells present at low precursor frequency *in vivo.* However, cells analysed after such procedures may no longer reflect those originally present *in vivo* because of the variable efficiency of outgrowth of different T cell subpopulations. To systematically assess this and to complement functional assays, we have analysed the TCR repertoire using a new high resolution RT-PCR method to determine TCR β chain CDR3 transcript length. In the *ex vivo* analysis of tumor infiltrating lymphocytes (TIL) of renal cell carcinoma and glioblastoma patients, we observed and quantified oligoclonally expanded populations of T cells that were very susceptible to repertoire modification upon subsequent *in vitro* culture with autologous tumor cells. This *in vitro* repertoire skewing occurred preferentially with TIL rather than peripheral blood lymphocytes and we noted that tumor cells rather than normal cells of the same tissue type were the most potent inducers of the effect. It was striking that this selection was sometimes negative: certain prominent T cell populations that were highly represented *in vivo* disappeared after *in vitro* re-stimulation. This suggests that the presentation of tumor associated antigens during culture may eliminate rather than enrich for *in vivo* primed T cells. It is clear that *in vitro* functional tests cannot adequately describe all T cells with tumor specificity. Approaches that allow the assessment of potentially antigen-reactive T cell populations *ex vivo* are thus an important advance in the global appraisal of anti-tumor T cell immune responses.

Introduction

T cell recognition of specific antigens restricted by MHC class I and class II molecules has generally been investigated using *in vitro* functional tests such as proliferation, cytotoxicity or cytokine release. However, such tests rely on (i) the availability of antigen (or cells expressing the antigen), (ii) a frequency of specific T cells appropriate for the sensitivity of the test and (iii) cells that are functionally competent *in vitro*. Such conditions are not always met from T cell populations isolated *ex vivo* and *in vitro* expansion or re-stimulation is thus frequently utilized. In the case of anti-tumor immune responses, mixed lymphocyte tumor cell culture (MLTC) is used to expand tumor cell-specific T cells in an *in vitro* environment that minimizes the immunosuppressive conditions that may have been originally present *in vivo*. This *in vitro* approach has sometimes led to the successful characterization of tumor-specific T cells (1), but has certain limitations. It probably underestimates the overall anti-tumor T cell response because T cells (particularly those infiltrating tumors) may have low proliferative potential (2), impaired cytolytic function (3) and defects in cytokine production (4) or could possibly be in an anergic state. Furthermore, in situations where purified antigens are not available (i.e. in most tumors), the tumor cells used for stimulating T cell cultures may not adequately present the putative antigens due to low or absent MHC expression (5–7), specific antigen loss (8), low antigen density (9,10) or defects in antigen processing (11).

In order to better assess the overall anti-tumor T cell response, we have taken advantage of a new high-resolution RT-PCR method based on the determination of the TCR β chain

Correspondence to: P.-Y. Dietrich

Transmitting editor: P. Kourilsky

Received 4 November 1996, *accepted* 7 April 1997

CDR3 transcript length (12,13). This technique is sensitive enough to detect T cell clonal expansions *in vivo*, without the necessity of functional tests and *in vitro* culture. Recurrent identical size transcripts are found after specific T cell clonal expansion in response to defined antigens (12–14). Their presence in tumor infiltrating lymphocytes (TIL) may thus reflect the response driven by putative and still undefined antigens. Indeed, we demonstrated that in certain cases, the T cell populations detected *in vivo* by these molecular methods could be expanded *in vitro* and were tumor specific in cytotoxicity assays (15).

In this study, we show that some significant clonal expansions detectable *in vivo* are lost after *in vitro* culture and that, furthermore, the fate of such populations is dependent upon the nature of the stimulating cells. Our results suggest that MLTC procedures utilized to expand antigen-specific T cells may instead induce the selective depletion of potentially antigen-reactive populations. Previous studies based on *ex vivo* phenotypic analysis of antigen-selected cells that express distinctive TCR also indicated that functional assays underestimate the real T cell response (16). The present data, mainly based on the analysis of CDR3 length profiles, demonstrate that new approaches are necessary to understand the *in vivo* significance of clonally expanded T cells that are non-functional or deleted *in vitro*.

Methods

Isolation and culture of tumor cells

Long-term tumor cell lines were established from renal cell carcinoma (RCC) and glioblastoma patients. Tumor biopsies were processed immediately after removal at surgery to obtain single-cell suspensions using enzymatic digestion. Briefly, tissue was enzymatically digested for 1–2 h at 37°C in culture medium containing 0.1% collagenase type IA, 0.002% DNase type II and either 0.001% hyaluronidase (for RCC) or 0.05% protease type I (for glioblastoma). The resulting cell suspensions were centrifuged over a Ficoll-Hypaque gradient (Pharmacia, Uppsala, Sweden) and then cultured in DMEM medium containing 10% FCS. All enzymes were obtained from Sigma (St Louis, MO) and media were from Gibco/BRL (Life Technologies, Paisley, UK). From one RCC patient, a normal kidney cell line that expanded for five passages was also obtained using the same techniques.

Isolation and culture of T cells

T cells were either obtained from digested tumor tissue as described above (i.e. TIL), from tumor draining lymph node (TLN) which was also digested in the same way or from peripheral blood lymphocytes (PBL) obtained after Ficoll-Hypaque centrifugation. For glioblastoma, non-adherent cells were taken from the tumor cell cultures described above after 3 days of culture.

For RCC patients, TIL and cells from TLN were expanded for 6 days prior to MLTC in RPMI 1640 with 10% human serum, 10 U/ml rIL-2 and 3% conditioned medium from phytohemagglutinin-activated lymphocytes. PBL were not expanded before MLTC. In MLTC for the first patient, $3–5\times10^5$ T cells (from TIL, TLN and PBL respectively) were mixed with 10^5 tumor cells in the presence of 10^6 irradiated LAZ 509 (Epstein–Barr virus-transformed cell line). Two stimulations at weekly intervals were performed (20- to 50-fold expansion between stimulations). For the second RCC patient, co-cultures of TIL were performed either with tumor cells or with normal renal cells, using the same conditions described above.

T cells derived from glioblastoma were cultured in Iscove's medium (Gibco/BRL) supplemented with 10% human serum and 30 U/ml rIL-2 to establish the TIL lines. Two re-stimulations were carried out at ~3 week intervals using allogeneic irradiated PBMC and 1 μg/ml purified phytohemagglutinin (Murex Diagnostics, Dartford, UK), and then a second MLTC (considering the first 3 days of culture to be the first MLTC) was carried out using 10^5 T cells with 10^4 autologous tumor cells (10- to 40-fold expansion between stimulations).

Flow cytometry

T cell populations and tumor cell lines were analysed on a FACScan equipped with Lysys II software, calibrated by eye (Becton Dickinson, Mountain View, CA). Each mAb was used at a saturating concentration predetermined by titration curves on positive cloned cell lines. Anti-TCRBV region mAb, JU74.3, E17.5F3, CAS1.1.3, Tamaya 1.2 (Immunotech, Marseille, France) and DE.4 (Institut Gustave Roussy, Villejuif, France) are directed against TCRBV13S6, BV17, BV14, BV16 and BV13S3 respectively. OKT3 (Ortho Diagnostics, Westwood, MA) reacts with the CD3 antigen; anti-NKTa mAb, recognizing an infrequent TCR αβ determinant (17), was used as a negative control. W6/32 (ATCC, Rockville, MD; HB95) and L243 (ATCC; HB55) recognize monomorphic determinants of HLA class I and class II gene products respectively. Staining was revealed with a FITC-conjugated goat anti-mouse Ig (Immunotech). All analyses were performed after gating on viable lymphocyte populations using a combination of forward and side light scatter (linear amplification).

TCRBV gene segment usage

TCRBV gene segment usage was determined by RT-PCR as previously described (18). Briefly, total RNA was prepared from tumor, lymph nodes, PBL and MLTC (0.2–0.5 g tissue or $2–5\times10^6$ cells) using TRIzol (Gibco/BRL) and converted to cDNA by standard methods using reverse transcriptase and an oligo d(T) primer. These cDNAs were amplified in non-saturating PCR conditions with a panel of experimentally validated 5′ sense primers specific for the 24 BV subfamilies and one 3′ antisense primer for the BC gene segment. The specificity of the amplified products was assessed after Southern blotting and hybridization with a labelled BC oligonucleotide, and also by verifying the length of the PCR products with values deduced from the BV1–24 and BC primer positions. The comparative analysis of each BV product between the different samples was achieved by densitometric analysis of the signals on the autoradiographs.

CDR3 size analysis of TCRBV transcripts

The CDR3 region of the PCR-amplified TCRBV1–24 transcripts was analysed using a run-off procedure, as previously described (12,13). Briefly, aliquots (2 μl) of the BV1–24/BC PCR products (40 cycles) were subjected to one to three

cycle run-off reactions, using dye-labelled oligonucleotide primers, specific either for BC or one of the 13 human functional BJ segments. The run-off products were then run on an automated sequencer in the presence of fluorescent size markers. The length of the DNA fragments and the fluorescence intensity of the bands were analysed with Immunoscope software (developed by C. Pannetier) or Genescan Analysis software (Applied Biosystems, Forster City, CA).

Sequencing of PCR products

TCRBV13–BC PCR products derived from glioblastoma samples were cloned into PBS-SK$^+$ vector (Stratagene, La Jolla, Ca). Competent XL-1 blue *Escherichia coli* (Stratagene) were transformed and plated for blue/white colour selection on media containing X-gal. Plasmid DNA was extracted from white colonies using the Qiagen Plasmid Mini Kit (Qiagen, Hilden, Germany) and sequenced using the Dye Terminator Cycle Sequencing Kit (ABI PRISM; Perkin Elmer, Forster City, CA) according to the manufacturer's instructions.

Quantification of clonal expansions in glioblastoma samples

To quantify the respective proportions of recurrent BV13 transcripts in the total BV13 mRNAs (Fig. 6), clonotypic primers hybridizing with the CDR3 region of the recurrent BV13 sequences from TIL *ex vivo* or after MLTC were synthesized and labelled with a 6-Fam fluorophore (GENSET, Paris, France). Primer sequences were 5′-AGCTCCCCGGTGTTCGGGAGT-3′ for the recurrent cDNA clone detected in TIL *ex vivo* (CDR3-*ex vivo* primer) and 5′-CTGCTCATTGTAAGTCCTCCA-3′ for the recurrent cDNA clone detected in TIL after MLTC (CDR3-*in vitro* primer). Samples were amplified using a BV13 and a BC primer, then aliquots of BV13–BC PCR products were subjected to an elongation with either a 6-Fam-labelled and nested BC primer (13) or with one of the two clonotypic primers. The two run-off products were loaded in equal amounts in the automated sequencer. The proportion of the specific sequence in the total BV13 mRNA population was then calculated by dividing the area under the curve (AUC) obtained with the clonotypic primers by the sum of the AUC obtained with the BC primer. This calculated ratio was then corrected by the relative specific activity of the clonotypic primers (see legend of Fig. 6). With the exception of sequencing, all molecular experiments were performed at least twice, with highly reproducible results.

Results

In previously published studies (15,19), the *in vivo* repertoire in RCC and the representation of a specific cytotoxic T lymphocyte (CTL) clone was analysed. Here, we extend these studies, to more globally and systematically analyse T cell repertoires *in vivo* and after *in vitro* culture. In particular we wished to address whether commonly used *in vitro* culture techniques always achieve the usual aim of expanding *in vivo* primed T cells. To circumvent the problem of non-detection of T cells without function in *in vitro* tests (or of cells that showed no further expansion *in vitro*), we used sensitive RT-PCR based methods of the TCR repertoire to analyse oligoclonal expansions of T cells. We have analysed blood (after Ficoll-Hypaque isolation) and unmanipulated tissue samples (RNA directly analysed *ex vivo*), and compared these to the corresponding T cells after various periods of *in vitro* culture.

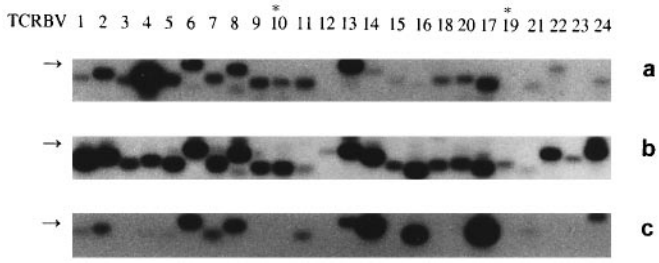

Fig. 1. Southern blot analysis of RT-PCR amplified TCRBV gene segments from TIL *ex vivo* (a), after 6 days culture with IL-2 (b) and after the second MLTC at day 20 (c) in RCC patient 1. The DNA fragments (0.25–0.5 kb) amplified by each BV–BC pair were hybridized with a BC oligonucleotide probe**.** PCR reactions were performed with the panel of primers described in (18) and results are presented according to the new nomenclature (55). Asterisks denote pseudogenes. Arrows indicate the 0.5 kb DNA marker position.

TCR repertoire of TIL analysed ex vivo is dramatically altered after in vitro culture with autologous tumor cells

TIL, TLN and PBL from RCC patient 1 were stimulated twice at weekly intervals using autologous tumor cells. Using RT-PCR, we analysed the BV gene usage in MLTC-derived T cell lines and compared it to that observed *ex vivo* from tumor, TLN or PBL. The amplified material was revealed by autoradiography. Results for TIL analysed *ex vivo*, after 6 days in culture with low-dose IL-2 alone or after MLTC are shown in Fig. 1. Each TCRBV gene product was obtained with the expected size (deduced from the positions of the TCRBV and TCRBC primers on cDNA sequence), varying from 250 to 535 bp. Autoradiographs were analysed by densitometry which allows the comparison of the usage of a given BV gene in the different samples, since the same primer pair was used in the PCR reactions (Fig. 2). The repertoire of T cells from uncultured PBL and TLN was diverse with almost all BV genes expressed, whilst T cells present within tumor displayed a restricted BV gene usage, with particularly abundant BV4 transcripts. In addition, the repertoire of PBL and TLN was not significantly modified by co-culture with autologous tumor cells. Indeed, the variation of the relative expression of any of the BV gene segments did not exceed the mean value $\pm$ 1 SD (20). In contrast, the same *in vitro* procedure induced major modifications of the BV gene usage in cultured TIL when compared with the *ex vivo* TIL repertoire, with a major increase in BV14, BV16 and BV17 expression, and a dramatic decrease in BV4 expression (Figs 1 and 2).

We also used TCRBV-specific mAb and flow cytometric analysis to confirm the results of the molecular analysis, and observed similar striking differences between the different co-cultures. In TIL after MLTC (comprising 92% CD3$^+$ cells), 45, 32 and 7% of lymphocytes were labelled with mAb to BV14, BV17 and BV16 respectively, whereas BV13 (mAb BV13S3 + BV13S6) T cells constituted $<$1.5% of the cells (Fig. 3). In contrast, none of these BV-specific mAb labelled $>$6% TLN lymphocytes in MLTC (data not shown). These

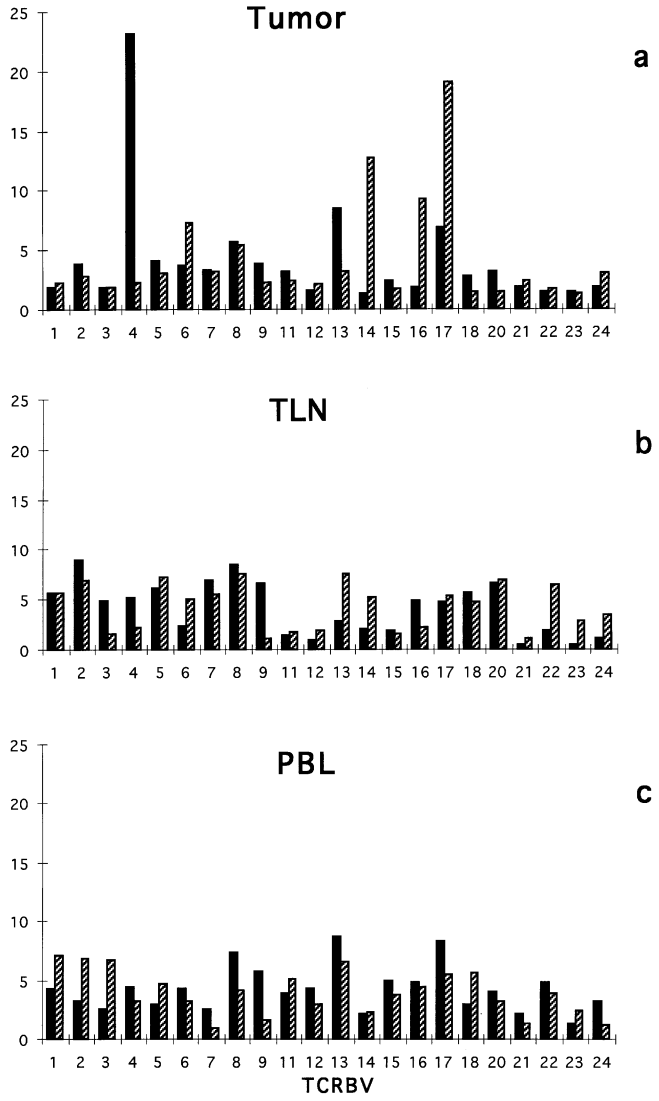

Fig. 2. Comparison of relative TCRBV gene segment expression in samples analysed *ex vivo* or after two rounds of MLTC in RCC patient 1. Tumor (a), TLN (b) and PBL (c) samples were analysed *ex vivo* (solid bars) or after MLTC (hatched bars). For each sample, the autoradiographic spots obtained by Southern blot of PCR amplified TCRBV gene segments (e.g. those obtained in Fig. 1) were analysed by densitometry and each BV signal was expressed as a percentage of the sum of all BV spots. Pseudogenes (BV10 and BV19) were not considered for this calculation. Values obtained for uncultured tumor, PBL and TLN have been previously reported (19). They are reproduced here to facilitate the comparison of patterns obtained before and after MLTC.

phenotypic data support the conclusion from the molecular analysis, i.e. there are high proportions of BV14, BV16 and BV17 T cells after *in vitro* stimulation with autologous tumor cells.

Oligoclonal TIL and TLN populations are subject to greater in vitro selective pressure than PBL

To assess the putative clonality of expanded T cells subsets in TIL after MLTC and their representation *in vivo*, we examined the CDR3 size distribution of the corresponding BV transcripts by run-off analysis of RCC samples (Fig. 4). BV–BC products were copied with a nested fluorescent BC and the sizes of the fluorescent run-off products were determined by electrophoresis on an automated DNA sequencer. Size variations of the run-off products are strictly due to different CDR3 lengths reflecting the imprecise V–D–J joining mechanism. In PBL of healthy donors, the repertoire of any BV–BC and BV–BJ combination usually displays a bell-shaped profile with 3-nucleotide-spaced peaks corresponding to in-frame sequences (13,21). In contrast, the emergence of one dominant peak reveals the presence of cDNAs with identical or same size in frame junctional regions.

Several observations can be made from these data. Some dominant peaks observed *ex vivo* disappeared or were significantly diminished after *in vitro* culture (Fig. 4). The BV4 transcripts, highly expressed in the *ex vivo* sample, contained one major peak corresponding to a CDR3 size of 10 amino acids (Fig. 4a). After MLTC (Fig. 4b), TIL BV4 transcripts contained a prominent peak not detected *ex vivo* (CDR3 size of 5 amino acids) and a weaker peak (CDR3 size of 10 amino acids) matching the main peak detected *ex vivo*. These data support the view of a counterselection of an *in vivo* expanded T cell population (with a 10 amino acid CDR3 size) during *in vitro* culture. Similarly, the intense peak (consistent with the expansion of T cells bearing a CDR3 of 8 amino acids) observed *ex vivo* in BV13 was not detectable after MLTC, whereas a peak corresponding to T cells with a 10 amino acid CDR3 size clearly emerged. The shift in the principal BV16 peaks (9 amino acids in *ex vivo* TIL sample versus 8 amino acids after MLTC) is an additional example (Fig. 4).

However, MLTC is clearly useful in some cases to facilitate the *in vitro* outgrowth of T cell populations that are detectable in *ex vivo* samples, but which do not represent major peaks. For example, $BV17^+$ T cells with CDR3 sizes of 10 or 11 amino acids that were present in the *ex vivo* tumor sample (but were not the predominant peaks) became the major populations after MLTC (Fig. 4). Furthermore, we have previously reported that one of these two peaks corresponded to a tumor cell-specific CTL clone obtained after direct cloning of TIL [(15), but referred to as TCRBV19 in this previous report according to old nomenclature]. Indeed, this same clone was also found to account for the peak corresponding to the CDR3 of 10 amino acids in TLN after MLTC (Fig. 4d).

In contrast to the significant selection observed in TIL after MLTC, the majority of BV–BC PCR products from PBL after MLTC conserved a bell-shaped pattern consistent with polyclonality (Fig. 4c), i.e. the same profile previously reported in uncultured PBL of RCC patients (19), possibly reflecting the lower frequency of tumor-specific T cells among PBL compared with TIL. The situation with TLN (Fig. 4d) was intermediate between that of PBL and TIL, with some polyclonal patterns conserved (e.g. BV4), but certain prominent peaks showing significant *in vitro* selection (e.g. BV17).

In vitro skewing occurs after culture of T cells infiltrating different tumor types

To see whether skewing phenomena were restricted to RCC, the TCR repertoire of T cells from human glioblastoma was also investigated. The BV gene segment usage of TIL after MLTC (10 days after the last addition of tumor cells and 50 days after

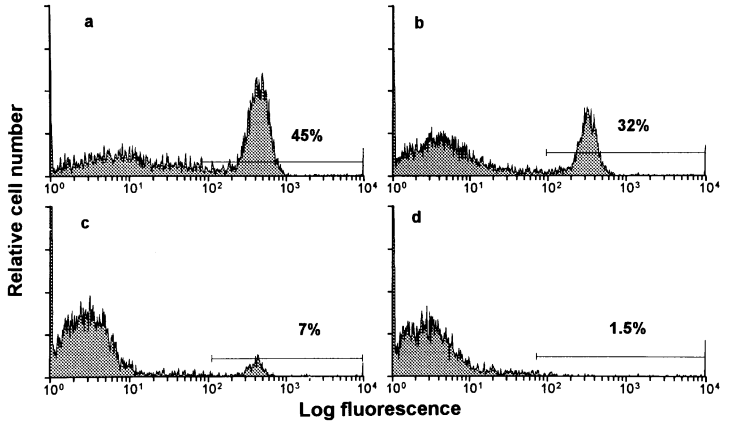

Fig. 3. Major expansion of certain TCRBV-expressing subsets in TIL after MLTC (RCC patient 1). Cells were stained with mAb to TCRBV14 (a), BV17 (b), BV16 (c), and BV13S3 and BV13S6 (d), and were analysed by flow cytometry. Histograms represent the relative number of cells versus intensity of fluorescence on a $\log_{10}$ scale.

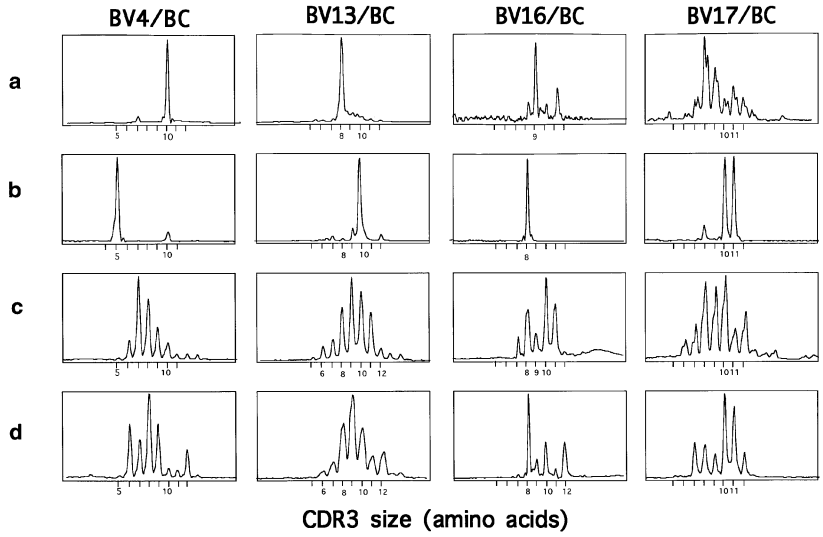

Fig. 4. Dramatic modifications in the CDR3 size distribution patterns of tumor BV–BC run-off products after MLTC (RCC patient 1). Total RNA from tumor sample *ex vivo* (a) or from MLTC T cell lines derived from TIL (b), PBL (c) and TLN (d) was reverse transcribed and amplified by PCR with BV and BC primers. Amplified cDNA was copied by a fluorescent BC primer in a run-off reaction and subjected to electrophoresis on an automated sequencer. The patterns obtained show the size and intensity distribution of in frame BV–BC amplification products. Horizontal axis: size in amino acids of the CDR3 region as defined in (56) and deduced from the fragment size. Vertical axis: fluorescence intensity, in arbitrary units. BV4–BC, BV13–BC and BV17–BC run-off products from the tumor sample *ex vivo*, and BV17–BC run-off products from the after MLTC sample have been previously reported [(19), but referred to as BV19 in this previous report according to old nomenclature]. They are reproduced here to facilitate the comparison of patterns obtained in the different samples.

the beginning of culture) was studied and compared with that observed in T cells *ex vivo* (tumor biopsy and PBL RNA: Fig. 5). Here again, some BV specificities highly expressed in TIL *ex vivo* disappeared after *in vitro* co-culture with autologous tumor cells (e.g. BV4, BV5, BV6 and BV15). In contrast, some BV specificities such as BV12 and BV16 poorly represented in the *ex vivo* sample gave rise to significant proportions of the T cell repertoire after *in vitro* culture. A further category can be considered: BV families that were highly represented when analysed *ex vivo* showing further expansion *in vitro* (e.g. BV13 and BV14). Of these subsets, $BV13^+$ T cells are of particular interest, since this BV is frequently overexpressed in glioma compared with PBL (22 and Dietrich *et al.*, manuscript in preparation). In the glioblastoma patient analysed here (Fig. 5), the relative expression of TCRBV13 transcripts was significantly higher in TIL *ex vivo* than in PBL. After subsequent *in vitro* culture of TIL, the TCRBV13 was the most abundantly expressed BV. Indeed, we confirmed the representation of $BV13^+$ cells in the cultured TIL by flow cytometry, which revealed $>70\%$ of total T cells reacting with mAb specific for BV13S1 and BV13S2 (data not shown). We therefore assessed whether the BV13 T cells expanding after *in vitro* stimulation with autologous glioma cells were the same as those overexpressed *in vivo*.

The run-off procedure was used to define the CDR3 size

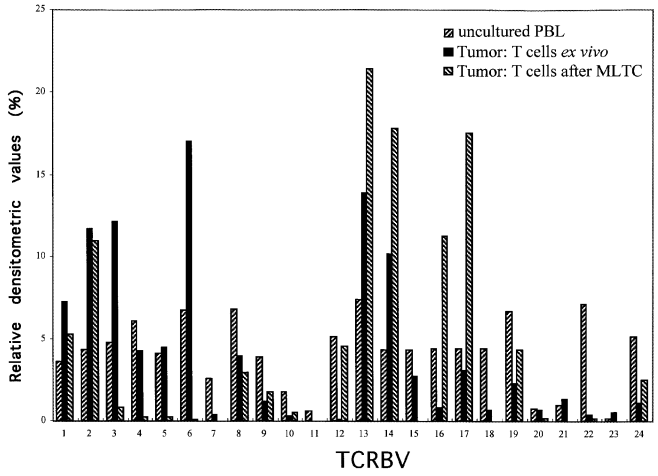

Fig. 5. Comparison of relative TCRBV gene segment expression in samples analysed *ex vivo* or after MLTC (day 50 after tumor dissociation) in a case of glioblastoma. For each indicated sample, the autoradiographic spots obtained by Southern blot of PCR amplified TCRBV gene segments were analysed by densitometry and each BV signal was expressed as a percentage of the sum of all BV spots.

profiles and the BJ usage of the BV13 population analysed *ex vivo* and after *in vitro* culture with autologous tumor cells (Fig. 6A). At a first level of analysis using the fluorescent BC primer, we observed that the BV13–BC PCR products from the *ex vivo* tumor sample displayed a nearly bell-shaped pattern with, however, two slightly prominent peaks corresponding to T cells bearing β chains with CDR3 sizes of 8 and 9 amino acids respectively. In the cultured TIL, a single peak (CDR3 of 8 amino acids) was highly predominant. To further characterize the BV13 transcripts with the same size CDR3 (8 amino acids), BV13–BC PCR products were copied with the 13 BJ primers. Each BV13–BJ profile of the tumor sample analysed *ex vivo* displayed several peaks corresponding to transcripts of different CDR3 sizes, often with an fluorescence intensity close to background levels (data not shown). The single exception was the BV13–BJ2S2 profile where an intense and prominent peak corresponding to a CDR3 size of 9 amino acids was noted. In contrast, no BV13–BJ2S2 amplified transcript was detected in TIL after MLTC, but a significant peak (CDR3 size at 8 amino acids) was seen in the BV13–BJ2S1 profile. Therefore, despite overexpression of BV13 T cells both *in vivo* and after *in vitro* culture, analysis of CDR3 size shows that the prominent BV13–BJ2S2 T cell population detected in the *ex vivo* sample did not expand *in vitro,* whereas the BV13–BJ2S1 T cells proliferating *in vitro* probably derive from minor *in vivo* populations.

Quantification of individual clones ex vivo and after MLTC

To quantify the extent of *in vitro* skewing, random sequencing of BV13–BC PCR products was used, leading to the identification of BV13 clones that were highly represented either in TIL *ex vivo* or in TIL after MLTC. A recurrent BV13S2–BJ2S2 sequence (four of eight sequences) with a CDR3 size of 9 amino acids was found in the *ex vivo* sample and a different recurrent BV13S1–BJ2S1 sequence (nine of 11 sequences) with a CDR3 size of 8 amino acids was found in TIL after MLTC (Fig. 6B). These data are consistent with the CDR3 profiles shown in Fig. 6(A). Clonotypic primers specific for the CDR3 region of both recurrent sequences were synthesized (CDR3-ex *vivo* and CDR3-*in vitro* primers: Fig. 6B). To determine the proportion of the BV13S2–BJ2S2 and BV13S1–BJ2S1 clones in the total BV13 population present in TIL *ex vivo* or in TIL after MLTC, the BV13–BC PCR products were copied with the labelled BC primer, the CDR3-*ex vivo* primer or the CDR3-*in vitro* primer and their CDR3 size distribution was analysed in the automated sequencer. The BV13S2–BJ2S2 clone represented 25% of the total BV13 mRNAs in the *ex vivo* sample (after correcting for the specific activity of the CDR3-*ex vivo* primer), but was undetectable in TIL after MLTC (Fig. 6C). Thus, this highly expanded clone that we estimate to represent ~4% of total T cells *ex vivo* (Figs 5 and 6) was eliminated in culture. On the other hand, the BV13S1–BJ2S1 clone remained undetectable in the *ex vivo* sample, whereas it was highly expressed in TIL after MLTC, representing 85% of the BV13 transcripts. Therefore, since the T cell line obtained after MLTC was comprised of 73% BV13S1$^+$ T cells (assessed by flow cytometry: data not shown), it can be estimated that this BV13S1–BJ2S1 clone represents a minimum of 62% of the T cell line. These data clearly demonstrate that the major *in vitro* expansion of T cells bearing a TCRBV13S1–BJ2S1 chain with a CDR3 of 8 amino acids derives from a population with minor representation *in vivo* [probably <1 in 10^5 T cells (21)].

In vitro skewing is most pronounced when tumor cells rather than normal cells of the same lineage are used for in vitro stimulation

Whilst TIL (rather than PBL) from different tumors were clearly susceptible to *in vitro* skewing, we wanted to determine if this was an intrinsic property of TIL in culture or whether different stimulator cells influenced this selection to a greater or lesser extent. We therefore compared the CDR3 profiles in three different cultures from another RCC patient: TIL cultured in the presence of IL-2 (Fig. 7a), TIL stimulated by autologous tumor cells (Fig. 7b) and TIL stimulated by autologous normal kidney cells (Fig. 7c). The representative profiles of BV–BC PCR products shown in Fig. 7 indicate that the co-culture of TIL with tumor cells induced a major restriction of the number of T cell clones within a given BV specificity, whilst no obvious difference was observed following co-culture with normal kidney cells. These results are consistent with tumor cell-specific signals to *in vivo* primed TIL that can act either in a positive (*in vitro* selection and growth) or a negative (inhibition of growth) manner. Unlike some tumor cell lines that are low or negative for MHC expression, the three tumor cell lines used for this study are MHC class I$^+$ (Fig. 8) and thus may potentially present antigens to class I-restricted CD8 T cells. Since MHC class II is not constitutively expressed (Fig. 8), the repertoire selection could thus be more pronounced for the CD8$^+$ T. However, we cannot exclude that cytokines secreted in the culture microenvironment may lead to the transient expression of MHC class II molecules, since class II expression was inducible by IFN-γ on the three cell lines (data not shown).

Discussion

Whilst high efficiency culture of normal human T cell populations (e.g. from PBL) is possible (23), the general experience

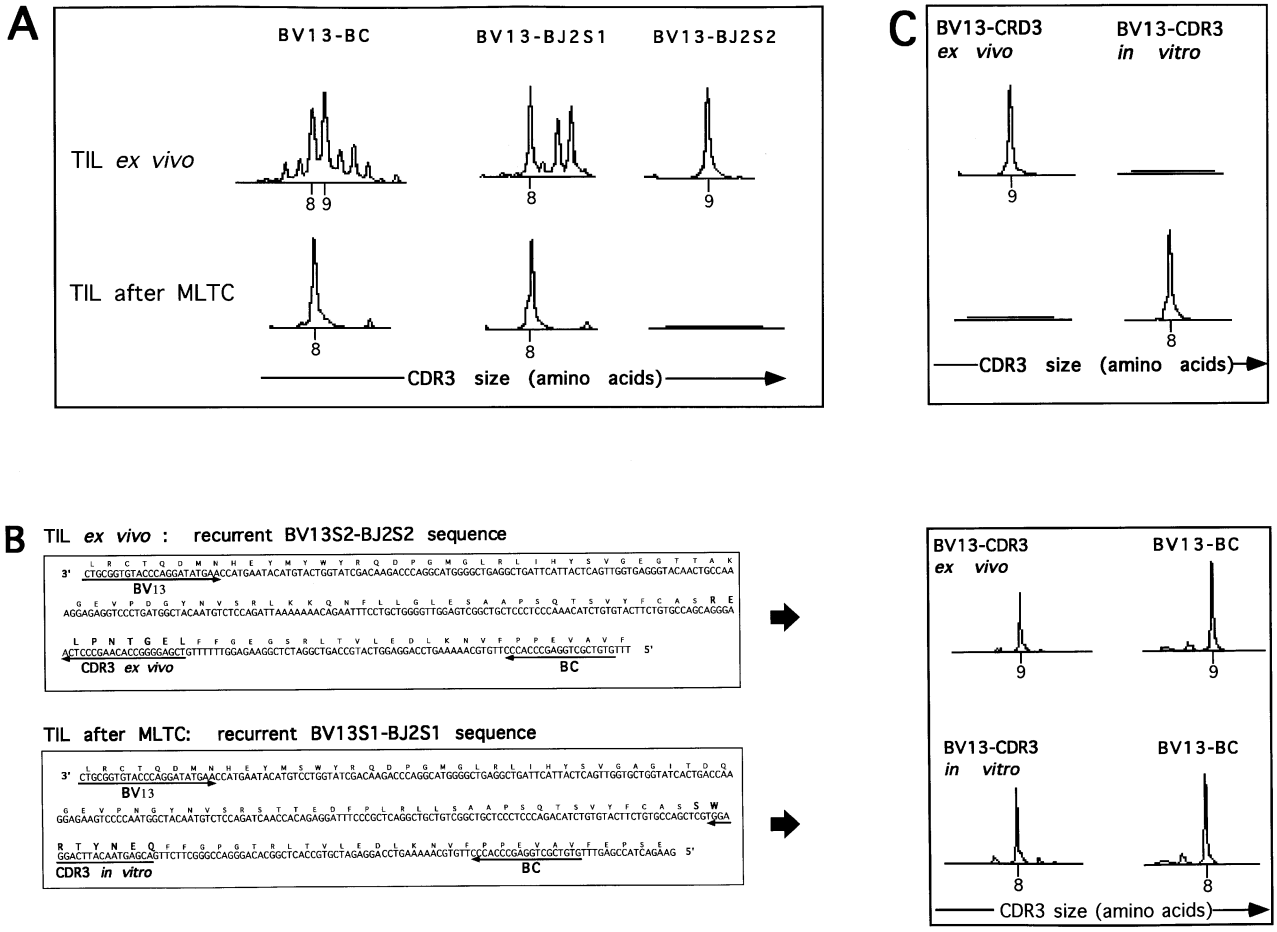

Fig. 6. Effect of MLTC on the representation of individual T cell clones in glioblastoma. (A) CDR3 size distribution patterns of BV13–BC, BV13–BJ2S1 and BV13–BJ2S2 run-off products from TIL analysed *ex vivo* or after MLTC. Amplified cDNA was copied by a fluorescent BC or BJ primer in a run-off reaction and subjected to electrophoresis on an automated sequencer. The patterns obtained show the size and intensity distribution of in-frame BV–BC or BV–BJ amplification products. Horizontal axis: size in amino acids of the CDR3 region. Vertical axis: fluorescence intensity, in arbitrary units. (B) Recurrent sequences found in TIL *ex vivo* (BV13S2–BJ2S2) or after MLTC (BV13S1–BJ2S1). BV13–BC PCR products from both samples were cloned into PBS-SK$^+$ vector and sequenced (see Methods). CDR3 sequences are in bold. The positions of primers (BV13, BC, as well as clonotypic primers CDR3-*ex vivo* and CDR3-*in vitro*) used for subsequent PCR are indicated on plasmid sequences. Plasmid DNAs were amplified using a BV13 and a BC primer. Equal amounts of the BV13–BC PCR products were elongated either with the BC primer or the clonotypic primer and their CDR3 size was analysed on an automated sequencer. The relative specific activities of clonotypic primers were then calculated by dividing the AUC obtained with the clonotypic primer by the AUC obtained with the BC primer (0.73 for CDR3-*ex vivo* and 0.78 for CDR3-*in vitro*). (C) Identification of the recurrent sequences in TIL *ex vivo* or after MLTC using the two clonotypic primers.

with T cells derived from cancer patients is of a much reduced clonogenic potential, particularly from TIL (2,24). Is this just because there is global immunosuppression affecting all T cells or are more specific inhibitory interactions in operation? This is an extremely difficult question to answer using traditional techniques to assess immune function and specificity, since only those cells that actually expand *in vitro* are testable. In an attempt to partially circumvent this technical limitation and to thus better define the overall anti-tumor immune response, we have exploited the high resolution properties of the analysis of CDR3 transcript length within *ex vivo* and *in vitro* populations defined for both BV and BJ usage (13). This technique generates data that reveal highly distinctive profiles corresponding to either polyclonal or oligoclonal T cell expansions. This has enabled us to identify T cell populations that were probably selected by antigen *in vivo* and to follow their fate systematically during culture. These results are thus not only descriptive of specific T cell populations, but are also predictive of TCR with potential antigen reactivity. Such predictions may therefore be exploitable for the subsequent isolation of T cell populations enriched for antigen specificity, as demonstrated in a case of chronic lymphocytic leukaemia (25). These approaches may be further refined by selecting subsets defined by other phenotypic markers (16).

Several features emerge from the present study. When lymphocyte populations were first analysed *ex vivo*, we observed a higher representation of oligoclonal T cell populations in TIL and to a lesser extent in TLN (i.e. those compartments in closest proximity to the tumor) compared with blood. However, the major finding of this study is that *in vitro* culture dramatically skews the representation of different T cell populations compared with their starting proportions. Whilst this was

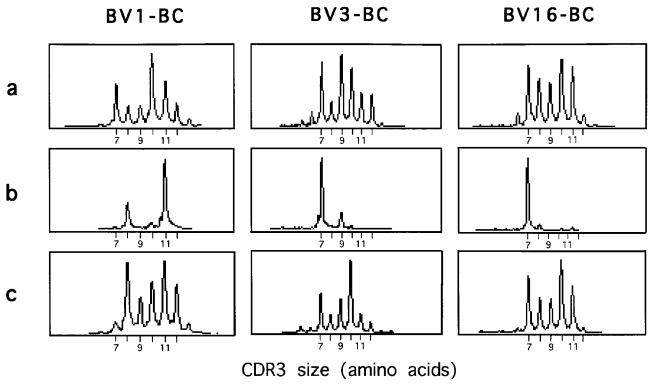

Fig. 7. Repertoire comparison for selected BV of TIL cultured in the presence or absence of different stimulator cells (RCC patient 2). CDR3 size distribution profiles are shown for BV–BC run-off products of TIL cultured without addition of stimulator cells (a), with autologous tumor cells (b) or autologous normal renal cells (c). Horizontal axis: size in amino acids of the CDR3 region. Vertical axis: fluorescence intensity, in arbitrary units.

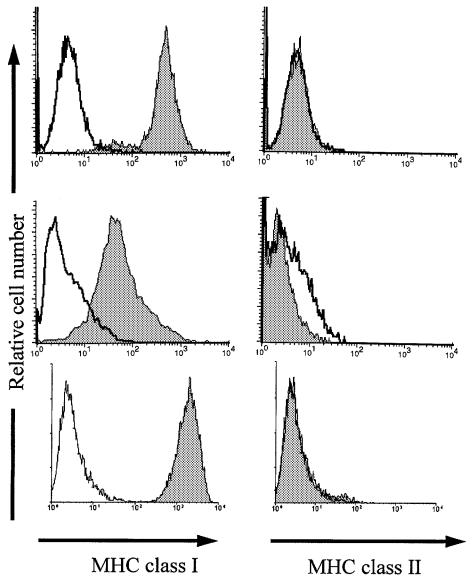

Fig. 8. RCC and glioblastoma cell lines constitutively express MHC class I molecules, but not class II. Cells were stained either with mAb to monomorphic determinants of HLA class I (W6/32) and class II (L243) molecules (filled curves) or an isotype-matched control mAb (open curves) and were analysed by flow cytometry. Upper histograms: RCC patient 1 cell line; middle histograms: RCC patient 2 cell line; lower histograms: glioblastoma cell line. Histograms represent the relative number of cells versus intensity of fluorescence on a $\log_{10}$ scale.

partly anticipated (since the principal objective of *in vitro* stimulation is to enrich for antigen specific cells), TCR analysis revealed that this *in vitro* selection was far from uniform. In particular, it was the highly oligoclonal TIL that were the most susceptible to repertoire modifications *in vitro*, especially after culture with tumor rather than normal cell lines. Indeed, the tumor cell lines studied here all expressed MHC molecules (constitutive for class I, inducible for class II), and thus are potential antigen-presenting cells (APC). This is consistent with a role of tumor-associated antigenic stimulation in this skewing, causing either *in vitro* expansion or elimination of T cells that were presumably primed *in vivo*. In our quantitative analysis we showed that MLTC can substantially expand clones poorly represented *in vivo*, which for one clone we estimated to be a 62,000-fold enrichment. Conversely, a major clone that we estimated to represent 4% of TIL *in vivo* disappeared after MLTC (Fig. 6). If the loss of oligoclonally expanded T cell populations is a specific effect, these eliminated populations warrant as much attention as T cell populations that actually proliferate in culture.

The *ex vivo* identification of T cell populations that subsequently exhibit highly diverse growth patterns during culture raises questions about their functional status at the outset. The cells that do expand *in vitro* are presumably in a primed state, responsive to the exogeneously supplied cytokines, particularly after re-stimulation by tumor cells. These populations are naturally the easiest to test for function and specificity, e.g. the $BV17^+$ cells that we discuss in this report that have been previously shown to specifically lyse RCC tumor cells [(15), but referred to as BV19 in this previous report according to old nomenclature]. In a case of a regressive melanoma, there is evidence that *in vitro* growth patterns may also depend upon T cell interactions. The growth of one CTL clone (BV13) with specific anti-tumor activity was achieved after MLTC, whereas another clone (BV16) with a higher representation *in vivo* could be cultured only after sorting, rather than as a bulk T cell line (26,27). Utilization of BV specific mAb for sorting is not always feasible since many specificities are not available and, furthermore, crude BV usage may not always provide sufficient enrichment for specific cells, as shown with our more refined analyses based on CDR3 size. However, it is clear that expression of an appropriate receptor for antigen recognition and *in vitro* growth are not sufficient conditions to ensure specific effector function. This may be due to selective defects in cytokine production (4) or impaired cytolytic function (3). Thus, we cannot exclude that, for example, the $BV14^+$ expanded T cells from RCC TIL (Figs 1–4) may be antigen specific, despite lack of specific cytolytic properties (15). Diminished or incomplete effector functions may be a consequence of immunosuppressive cytokine secretion in the tumor microenvironment, such as transforming growth factor-β or IL-10 (28–34), or by the expression of FasL on the tumor cell surface (35–38). Alternatively, inadequate expression of co-stimulatory molecules by tumor cells used for stimulation may lead to only partial T cell activation (39–41). A further hypothetical possibility is that the tumor cells present modified antigenic peptides that still engage the same TCR but that elicit qualitatively different T cell responses (42–45). Such mechanisms would be consistent with the oligoclonal T cell expansions observed *ex vivo* in RCC (19) and glioblastoma (22), and also in other malignancies (25,46), but the lack of spontaneous tumor rejection in most cases.

It is clear from some experiments that certain T cells poorly represented *in vivo* strongly expand *in vitro*. This may reflect the growth of tumor antigen-specific cells now able to proliferate in the absence of the immunosuppressive conditions that were present *in vivo*: these cultures therefore achieve one of the principal goals of MLTC. Indeed, the *in vitro* expansion of CTL precursor cells has successfully led to the identification of the majority of tumor-associated antigens characterized to date. However, caution must be exercised in interpreting *in vitro*

defined T cell specificities. Cultured tumor cells used for *in vitro* stimulation do not necessarily reflect the majority of those present *in vivo* and the hierarchy of antigenic peptides presented may be altered. It is also possible that totally new epitopes are expressed by outgrowth of tumor variants or indeed may be derived from proteins used in culture (e.g. bovine serum). However, these possibilities assume either cross-stimulation of T cells previously activated *in vivo* by other peptides or *in vitro* priming. This latter possibility is only likely to be achieved by very specific culture conditions, such as stimulation with peptides with high affinity to MHC class I (47), high peptide density (10), B7 expression (48) or particular cytokine cocktails (49,50). Finally, mitogens may activate irrelevant bystander T cells, but this would not explain the selective skewing observed in TIL subjected to tumor cell co-culture, unless previously antigen-primed cells are differentially responsive to mitogenic stimulation.

The final category of T cells is perhaps the most tantalizing: the prominent oligoclonally expanded populations detected *ex vivo* that are eliminated or not detectable after *in vitro* culture. These cells are thus totally invisible to any immunological test carried out on cultured T cells. They may be specifically deleted by tumor cell contact *in vitro* which could be the *in vitro* correlate of specific immunosuppression by tumor cells *in vivo*. It may therefore be fruitful to target *in situ* analyses on the T cell populations that disappear *in vitro* to search for cells in the process of being eliminated, e.g. cells undergoing apoptosis.

In conclusion, *in vitro* tests of T cell function and specificity are useful indicators of immune responses when positive results are obtained, but are clearly insufficient to quantitatively or qualitatively describe the *in vivo* immune response. The underestimation of T cell involvement in immune responses when assessed by cytotoxicity assays alone has been suggested in independent studies using different approaches. In a class I-restricted response to a defined peptide antigen, in which cells bearing specific TCR could be identified phenotypically *ex vivo*, very high proportions (up to 80%) of potentially antigen-reactive T cells were detected in certain subsets (16). This was in contrast to other murine studies in which CTLp frequencies determined by classical limiting dilution analysis rarely exceeded 1–2% of a defined CD8 subset (51–53). Moreover, in human clinical studies, totally negative *in vitro* tests of CTL function were obtained from HLA-A1$^+$ melanoma patients immunized with the well-characterized HLA-A1 binding MAGE-3 peptide, despite tumor regression in certain patients (54).

The potential range of T cell effector functions is not fully understood in either its diversity or its regulation. Furthermore, it is clear that effector function is not expressed in a stable or irreversible manner. The most stable and conserved parameter in specific antigen recognition is the expression of the TCR, we thus suggest that assessing TCR usage will be an essential component in globally describing an immune response.

Acknowledgements

This work was supported by the Swiss National Foundation (no. 31-40704.94), la Ligue Genevoise contre le Cancer (P. R. W.), la Fondation de France (P. S.), and les Fondations Terwindt, MEDIC and Spinola. We would like to thank Catherine Metral for her expert technical help, Christophe Pannetier for helpful comments, and Pierre Alberto for continuous support and encouragement. We acknowledge the excellent secretarial assistance of Melissa Morawitz. We thank the editors of *Cancer Research* for permission to reproduce certain CDR3 profiles previously published.

Abbreviations

APC	antigen-presenting cell
AUC	area under the curve
CDR	complementarity determining region
CTL	cytotoxic T lymphocyte
MLTC	mixed lymphocyte tumor cell culture
PBL	peripheral blood lymphocyte
PBMC	peripheral blood mononuclear cell
RCC	renal cell carcinoma
TIL	tumor infiltrating lymphocyte
TLN	tumor draining lymph node

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