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J Clin Invest. 1994;**93**(4):1397-1402. <https://doi.org/10.1172/JCI117116>.

Research Article

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Direct Evidence to Support the Immunosurveillance Concept in a Human Regressive Melanoma

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Abstract

The concept of immunosurveillance against cancer has been an extensively debated question over the last decades. Multiple indirect arguments have supported the view that the immune system may control, at least in certain cases, malignant cell growth while direct demonstration is still lacking in the human. In an attempt to address this issue, we have selected a study model, namely spontaneously regressive melanoma. In previous series of experiments, the variability of T cell receptors (TCRs) in the lymphocytes infiltrating a regressive tumor lesion was investigated. Results demonstrated that clonal T cell populations, precisely defined through their V-D-J junctional sequences, were amplified in situ. One clone was predominant, expressing the V β 16 variable gene segment. A specific anti-V β 16 TCR mAb was generated here to purify and functionally characterize the corresponding cells. A tumor-infiltrating lymphocyte-derived V β 16⁺ T cell line was developed using this reagent. These in vitro cultured cells were found to express the in vivo predominant TCR sequence exclusively and to display an HLA-B14-restricted cytotoxic activity against the autologous tumor cells. Immunohistochemical experiments, performed with the anti-V β 16 mAb, showed that the corresponding CTLs are present in the tumor area, some of them being closely opposed to the melanoma cells. Together, these studies demonstrate the existence of a local adaptive immune response clinically associated to tumor regression, thus strongly supporting the validity of the immunosurveillance concept in certain human tumors. (*J. Clin. Invest.* 1994. 93:1397-1402.) **Key words:** melanoma • lymphocytes • tumor infiltrating • neoplasm regression • spontaneous

Introduction

Previously, we have studied a case of melanoma displaying the well-agreed clinical and histological characteristics of "spontaneous" tumor regression (1). It was initially shown by immunohistochemical experiments that T cell receptor (TCR)¹ α/β ⁺ lymphocytic infiltrates were present in the regressive lesion

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Received for publication 31 August 1993 and in revised form 8 December 1993.

1. Abbreviations used in this paper: TCR, T cell receptor; TIL, tumor-infiltrating lymphocytes.

(1). PCR-based molecular analysis of the TCR β chains expressed by the tumor-infiltrating lymphocytes (TIL) was performed directly in situ demonstrating the overrepresentation of a limited number of V β gene segments, in particular V β 16 (22 vs. 4% in PBL). Extensive sequence analysis of the V-D-J junctional regions of the V β 16 transcripts expressed in situ showed that 84% of them corresponded to a unique mRNA, while the control V β 16 sequences from autologous PBL were polyclonal (1). These experiments demonstrated that clonal T cell populations were indeed selected and amplified at the tumor site, but were not informative regarding the function of the corresponding lymphocytes. It was subsequently attempted to characterize the V β 16⁺ T cells through in vitro generation of cell lines and clones derived from the TIL. This attempt failed although we could characterize other T cell clones overgrowing in the culture while being less dominant in vivo (2). Note that one of them, expressing the V β 13 gene segment, was shown to display high levels of cytotoxicity against the autologous tumor cells (2). Why the cell type most represented (i.e., V β 16⁺) in vivo displayed a relatively poor capacity of expansion in vitro remains unclear.

In an attempt to circumvent this difficulty and characterize the V β 16 cells, we have generated here a specific anti-V β 16 TCR mAb to allow positive selection and specific proliferative triggering of the corresponding cells. Using this approach, we could demonstrate that the V β 16⁺ lymphocytes represent a unique HLA class I-restricted cytotoxic T cell (CTL) strongly active against the autologous tumor. In addition, immunohistochemical analysis with the anti-V β 16 mAb provide evidence that the corresponding clone is indeed present in the regressive melanoma lesion. Together, the results demonstrate that V β 16⁺ T cells, found to be predominant in situ, display a tumor-directed cytotoxic effector function in the clinical context of cancer regression.

Methods

Patient. The patient under study was a 65-yr-old woman suffering from a primary malignant melanoma with clinical and histological signs of tumor regression, as described previously (1).

TIL cultures. Generation of the TIL-derived T cell line from the regressive melanoma lesion was described previously (2). Cells were expanded by plating 5×10^3 cells per well in 96-well, V-shaped microtiter plates (Nunc, Roskilde, Denmark) in the presence of irradiated autologous EBV-B cells (60 Gy, 1.5×10^4 cells/well) plus allogeneic PBL (35 Gy, 7×10^4 cells/well) and in the presence of 100 U/ml human recombinant IL-2 (Roussel-Uclaf, Romainville, France), and 3% T cell growth factor.

Tumor cell lines. Generation and expansion of the autologous melanoma cell line (M10) has been described previously (2). Analysis of the HLA phenotype of this cell line revealed the same specificities (HLA-A3, -A10, -B14, and -B35) found on autologous PBL (data not shown). Tumor lines were maintained in monolayer and passaged

J. Clin. Invest.

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Volume 93, April 1994, 1397-1402

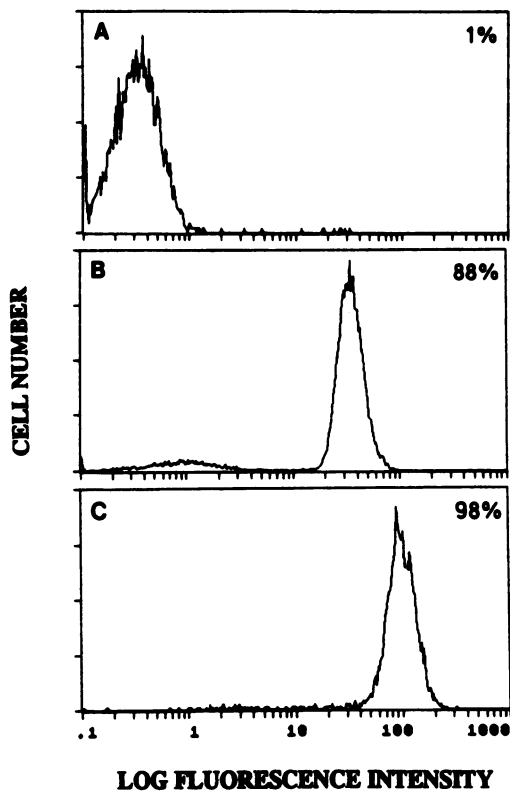


Figure 1. Flow cytometric analysis of the V β 16⁺ cells stained with anti-V β 16 mAb before (A), after first (B), and after second (C) round of immunomagnetic separation. Histograms represent the number of cells versus intensity of fluorescence on a logarithmic scale. The percentage represents the amount of V β 16⁺ cells compared with the control mAb NKTa.

with 0.05% trypsin. Additional tumor cell lines maintained in culture include two human allogeneic melanoma cell lines (M11, M12). Immunofluorescence analysis with antibodies to HLA-A3, -A10, -B14, and -B35 demonstrated that these allogeneic cell lines do not share an HLA class I antigen with the M10 autologous cells (data not shown). K562 is a human myeloid leukemia line commonly used as a target for natural killer cells. LAZ 388 is an EBV-transformed B cell line described previously (2). LCL is an EBV-transformed B cell line derived from autologous PBL.

mAbs, serological reagents, and phenotypic analysis. 145-2C11 (2C11) recognizes the ϵ chain of the murine CD3 molecule (3). BMA 031, kindly provided by Dr. R. Kurrle (Behring Company, Marburg, Germany), reacts with a monomorphic determinant of TCR α/β receptor (4). Anti-TCR- δ 1 mAb (T Cell Science, Cambridge, MA) recog-

nizes a constant epitope of the human TCR- δ chain (5). OKT3, OKT4, and OKT8 (Ortho Diagnostics Systems, Inc., Westwood, MA) react with the CD3, CD4, and CD8 proteins, respectively. W6/32 (6) mAb recognizes a monomorphic determinant of HLA class I gene products. Anti-NKTa mAb recognizes an infrequent TCR α/β clonotypic determinant (7). Each mAb was used at a saturating concentration predetermined by titration curves on positive cloned cell lines.

Human HLA-reactive pregnancy sera (anti-A3, anti-A10, anti-B14, anti-B35) were kindly provided by Dr. C. Raffoux (Hopital Saint Louis, Paris, France). Single- and two-color indirect immunofluorescence analyses were performed using an Elite flow cytometer (Coulter Electronics Inc., Hialeah, FL) as described previously (7).

Generation of mAb against human V β 16 gene segment. Production of a V β 16 transfectant cell line was performed as described by Romagné et al. (8). Briefly, cDNA was prepared from PBL and submitted to PCR amplification using a C β -specific antisense primer (8) and a V β 16-specific sense primer designed to introduce an EcoRI restriction site as well as the CCACC Kozak sequence (9) in front of the AUG codon (5'-GCGAATTCACCATGGTTTCCAGGCTTCTCAGTT-3'). Amplified products were cloned into the pBSmC β 215 vector (8). After subsequent XhoI-NotI double restriction digest the hV β 16-mC β fragment was subcloned into the NT expression vector (F. Romagné, unpublished results) corresponding to pSV2neo with insertion of the SR α promoter (10). The recombinant construct (V16NT) was then electroporated into DOIS-19 cells (11) together with the pCA 258 plasmid for high yield coexpression of the CD3zeta chain (12). After selection for G418 resistance TCR-positive cells were subsequently identified by flow cytometry as described (8).

BIOZZI mice were immunized with transfectants expressing a chimeric TCR β chain that includes the hV β 16 segment (13). Spleen cells from the immunized animals were then fused to the NS-1 nonsecreting myeloma (14). Supernatants of growing hybridoma cells were screened for the ability to produce an anti-V β 16 mAb as described by Romagné et al. (8).

Purification of V β 16⁺ cells from the TIL-derived T cell line. V β 16⁺ cells were purified from the TIL cell line by positive selection with magnetic beads (Dynabeads; Dynal, Oslo, Norway). For separation, cells were stained with anti-V β 16 mAb (1:400), a saturating concentration predetermined by titration curves on a V β 16⁺ IL-2-dependent cell line, and incubated with Dynabeads M-450 sheep anti-mouse IgG (Dynal) with a ratio of three beads for one estimated positive cell. Positive cells were then separated using a magnet.

Immunohistochemical staining procedures. Anti-V β 16 mAb was used at 1:50 dilution selected after a series of preliminary experiments performed to assess the optimal experimental conditions. As controls we have used several mAbs such as pan-TCR α/β reagent (BMA 031 mAb), pan-TCR γ/δ reagent (anti-TCR δ 1 mAb), and other mAbs against T cell-associated antigens (CD3, CD4, CD8). A polyclonal biotinylated sheep antibody to mouse Ig (reactive with all mouse isotypes) and a streptavidin-biotinylated peroxidase complex (all obtained from Dako, Glostrup, Denmark) served as a detection system for the primary antibody. 3-Amino-9-ethylcarbazole (Dako) was used

Table I. Analysis of TCR V β 16 and V α 4 Chain Junctional Sequences in V β 16⁺ Cells

V region					N region						J region				
Vβ16	C	A	S	S	L	R	D	S	W	N	Y	E	Q	F	Jβ2.1
	TGT	GCC	AGC	AGC	CTC	CGG	GAC	TCG	TGG	AAC	TAT	GAG	CAG	TTC	
Vα4	V	Y	Y	C	I	P	Q	G	G		D	D	W	G	JGermd
	GTG	TAC	TAT	TGC	ATC	CCT	CAG	GGG	GGT		GAC	AGC	TGG	GGG	

cDNA from the V β 16⁺ cell line was amplified with a V α 4- or V β 16-specific 5' oligonucleotide primer, paired with the corresponding C α - or C β -specific 3' primer. PCR-generated V β 16 and V α 4 TCR chain fragments were cloned into a EcoRV-cut ddT-tailed pBS-SK⁺ vector. Double-stranded plasmid DNAs were sequenced directly with the Sequenase kit (United States Biochem. Corp.). A translated amino acid sequence is shown above each corresponding nucleotide sequence.

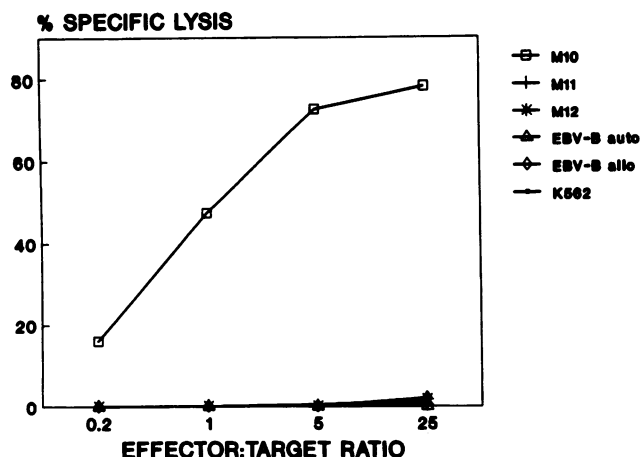


Figure 2. Specificity of V β 16⁺ cells directed against autologous melanoma cells. Cytotoxicity was determined in a 4-h ⁵¹Cr release assay on autologous (M10) and allogeneic (M11, M12) melanoma cell lines, autologous and allogeneic EBV-transformed B cells, and the NK target K562, as described (2). Values represent means of triplicates (SD < 5%) at E/T ratios of 25:1, 5:1, 1:1, and 0.2:1.

as substrate for the enzyme; the peroxidase reaction resulted in an intense red precipitate.

Cytotoxicity assays. The cytotoxic activity of the V β 16⁺ TIL cell lines was measured by a conventional 4-h ⁵¹Cr release assay using triplicate cultures in V-bottomed plates. E/T ratios were 25:1, 5:1, 1:1, 0.2:1, and 0.04:1 on 2,000 target cells per well. The percentage of specific cytotoxicity was calculated conventionally; SDs were < 5%.

Functional effects of the antibodies and antisera, either on effector cells (OKT3, OKT4, OKT8, BMA031, TA-37) or on target cells (W6/32, antisera anti HLA-A3, anti HLA-A10, anti HLA-B14, anti HLA-B35), were tested by incubating each of them for 2 h at 37°C before the assay at the predetermined saturating concentration. The percentage of inhibition of lysis was calculated as:

$$1 - \frac{\% \text{ specific lysis in mAb/antisera-treated wells}}{\% \text{ specific lysis in control wells}} \times 100.$$

Synthesis of the first strand of cDNA. Total RNA was prepared from the V β 16⁺ cell line using a single-step guanidinium isothiocyanate-phenolchloroform extraction method (15). First-strand cDNA was synthesized with oligodeoxythymidine (oligo-dT) priming and reverse transcriptase.

Molecular analysis of TCR V gene segments using PCR. The procedure used to analyze TCR V α and V β gene segment expression has been described previously (16). Briefly, these TCR transcripts cDNA were amplified in a 30-cycle PCR, using the 29 different V α and 24 V β 5'-oligonucleotide primers (V α 1-w29/V β 1-w24), each of them paired with the corresponding C α - or C β -specific 3' primer. Positive α and β controls consisted of constant region amplifications (180 and 190 bp, respectively) and negative controls were performed without cDNA (16). The amplified products were detected by Southern blot analysis using a C α or C β oligonucleotide probe.

Cloning and sequencing of V β 16 and V α 4 transcripts. The technique used for cloning and sequencing of V α and V β transcripts has been described previously (1). Briefly, the primers for the cloning of V β 16 sequences were 5'-GCCTGCAGAACTGGAGGATTCTGG-3' in the V region and 5'-GGTGTGGGAGAATTCTGCTTCTGA-3' in the C region. For amplification of V α 4 sequences the primers were 5'-TTGGTATCGACAGCTTCACTCCA-3' for V region and 5'-GTTGCTCCAGGCCGCGGCACTGTT-3' for C α region. The amplifications were performed in 1 round of 30 cycles. After ethanol precipitation, the amplified products were separated on a 2% agarose gel and purified by absorption on glass beads (Gene Clean; BIO 101, Inc., La

Jolla, CA). The material was directly ligated into a EcoRV-cut ddT-tailed pBS-SK⁺ cloning vector (Stratagene, La Jolla, CA), as previously described (17). Competent XL-1 blue *Escherichia coli* strains (Stratagene) were transformed and plated for blue/white color selection on media containing X-Gal. The white colonies were screened by the dot blot technique and a C β or C α oligonucleotide probe. Plasmid DNA was extracted from positive colonies and sequenced by the di-deoxy-chain termination procedure (Sequenase 2.0; United States Biochem. Corp., Cleveland, OH).

Results

Generation of a V β 16⁺ cell line derived from the TIL population. A plasmid, containing the V β 16 sequence expressed predominantly in the TIL in vivo (1), was linked to the murine C β gene and electroporated into DOIS 19 cells, a TCR- β ⁻ variant of the mouse T cell hybridoma DO-11.10.7 (8, 11). Mice were immunized with a stable transfectant expressing the corresponding TCR chain. Hybridomas were screened by flow cytometry using transfected vs. nontransfected cells. The specificity of one of them (TA-37) was established according to a series

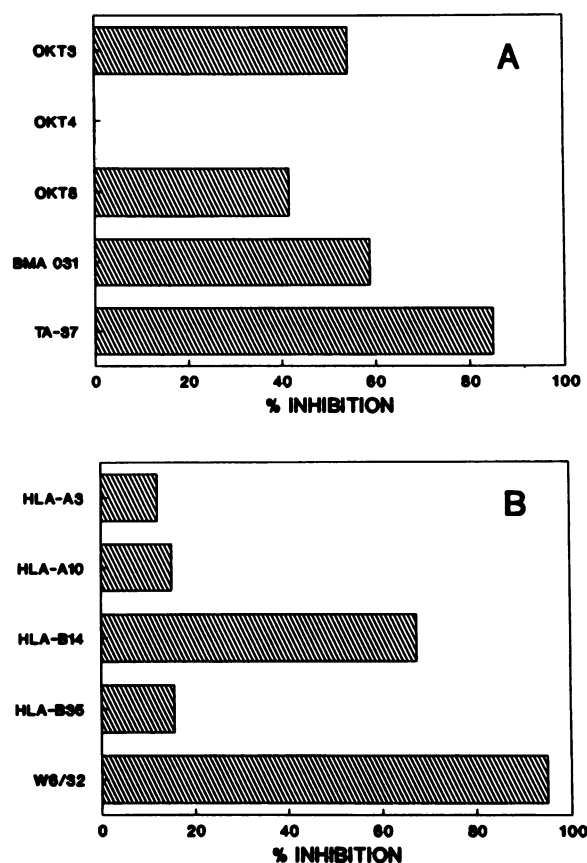
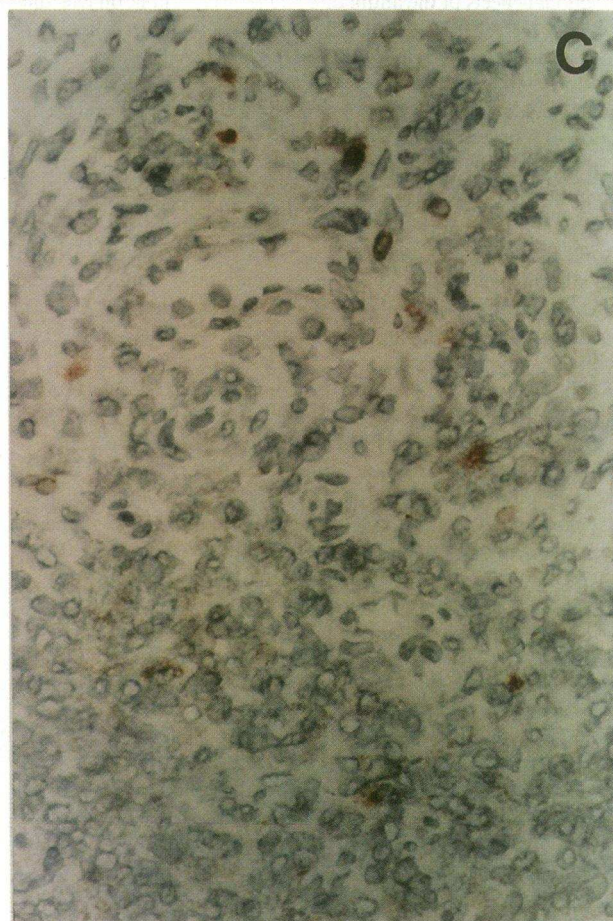
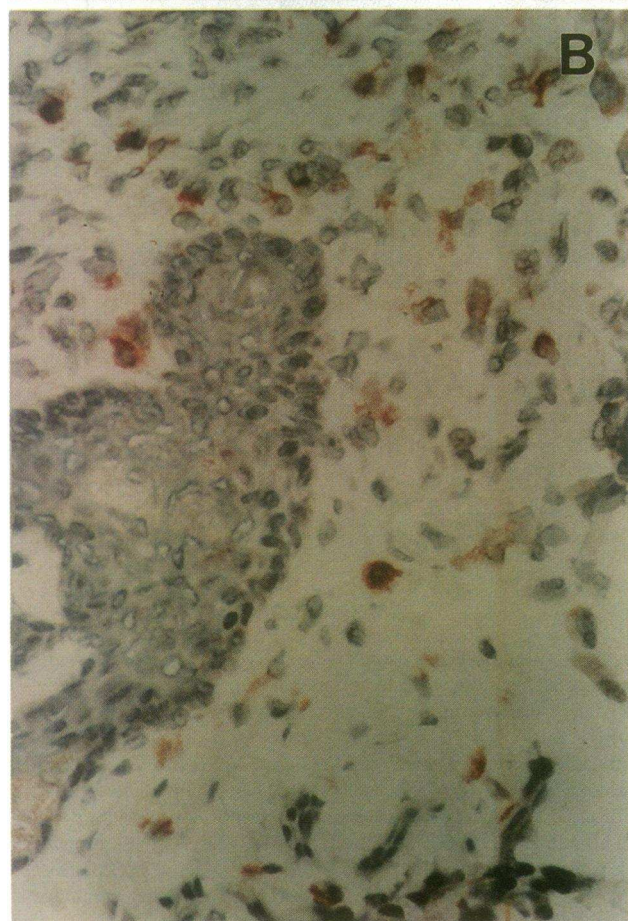
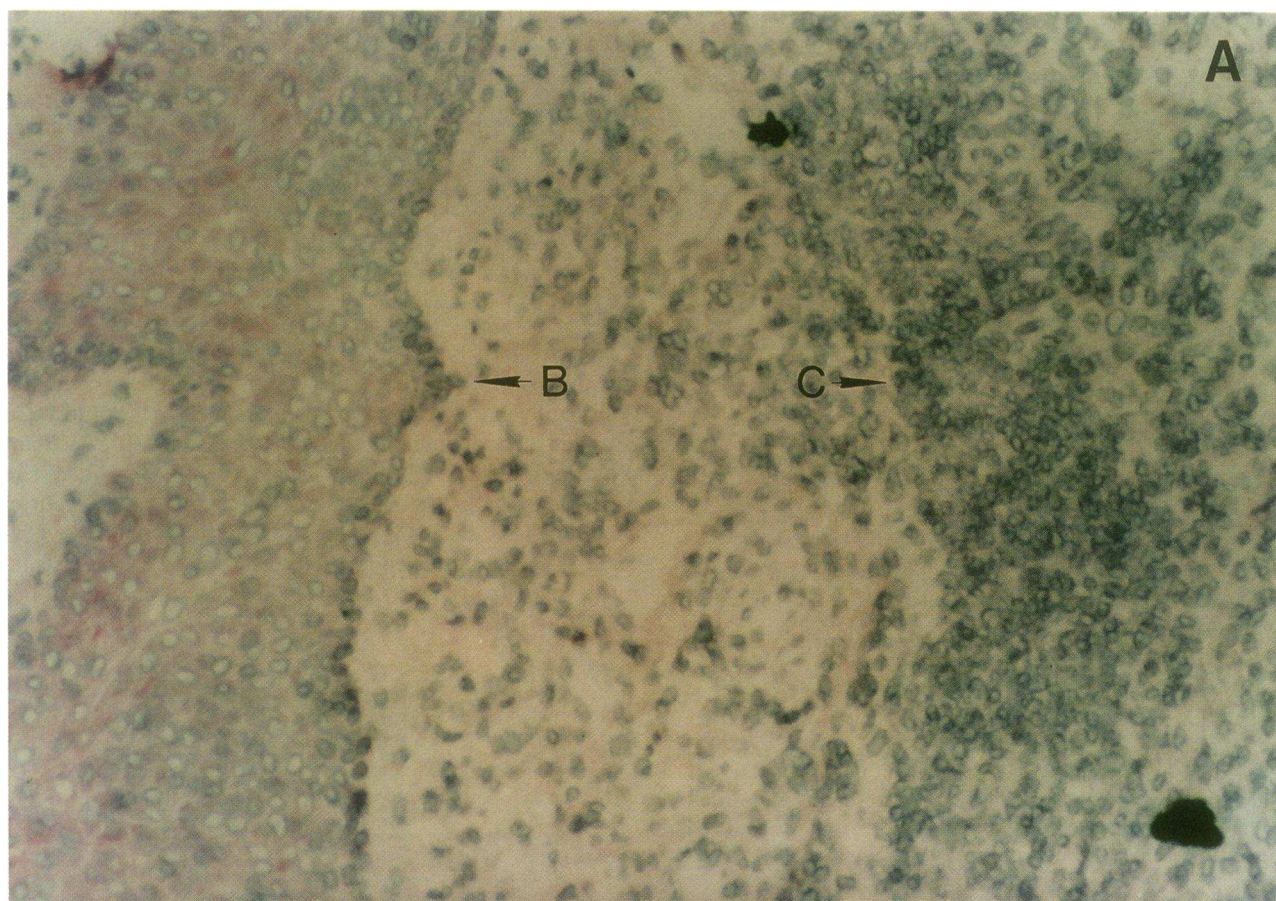


Figure 3. Inhibition of specific cytotoxicity of V β 16⁺ cells towards autologous melanoma cells by monoclonal antibodies and antisera. (A) Effector cells were preincubated with each of the antibodies (OKT3, anti-CD3; OKT4, anti-CD4; OKT8, anti-CD8; BMA031, anti-TCR α/β monomorphic; TA-37, anti-V β 16) for 2 h before the cytotoxicity assay. (B) Target cells (M10) were preincubated either with mAb W6/32 (anti-HLA class I monomorphic) or with polyclonal human antisera to HLA-A3, -A10, -B14, -B35 at 1:2 dilutions for 2 h before the addition of the effectors. Cytotoxicity was determined in a 4-h ⁵¹Cr release assay at an E/T ratio of 1:1, and the percentage of inhibition was calculated as described before (2).



of previously described procedures (18). In particular, purified TA-37⁺ cells from peripheral blood of normal donors were shown to express V β 16 transcripts exclusively while displaying a highly polymorphic TCR V α repertoire (data not shown). TA-37 mAb reacts with 2–4% of peripheral CD3⁺ T lymphocytes in healthy individuals (data not shown).

As expected from previous studies (2), the IL-2-dependent TIL cell lines derived from the regressive lesion after stimulation with autologous tumor cells and/or autologous EBV-transformed B cells (EBV-B) were found to contain ~ 1% TA-37⁺ cells (Fig. 1 A). One of them was subjected to several cycles of purification with TA-37 (using immunomagnetic beads) and subsequent culture with IL-2. The cell line obtained after 10 d of additional expansion consisted of 88% V β 16⁺ cells (see Fig. 1 B). The purity was eventually increased up to 98% V β 16⁺ cells through a second round of immunomagnetic separation (Fig. 1 C).

Flow cytometry analysis of this cell line showed a CD2⁺, CD3⁺, CD4⁺, CD8⁺, CD56⁺, TCR α/β ⁺, TCR γ/δ ⁺ phenotype (data not shown). TCR V α and V β gene segment subfamilies expressed in the cells were analyzed after PCR amplification with a series of previously described TCR-V subfamily-specific oligonucleotides (16). These T lymphocytes were found to express a unique V β subfamily, namely V β 16, as well as a unique V α 4 gene segment (data not shown). Characterization of the V β 16 transcripts showed the presence of a unique V β 16-LRDSWN-J β 2.1 junctional sequence (Table I), corresponding to that found in 38 of 45 cDNA tested from the V β 16 mRNAs expressed in situ (1). Sequence analysis of the V α 4 gene segment also revealed the presence of a unique transcript with a V α 4-IPQGG-JGermd junctional sequence (Table I). Together, these data demonstrated that the cell line was made of a unique V β 16⁺/V α 4⁺ T lymphocyte, which does correspond to the clone selected and expanded at the tumor site.

Functional characterization of the V β 16⁺ TIL-derived T cell line. The V β 16⁺ cells were tested in standard ⁵¹Cr release assay against a panel of autologous and allogeneic target cells. As shown in Fig. 2, V β 16 cells displayed high levels of cytotoxicity (> 70% specific lysis at an E/T ratio of 5:1) against the autologous melanoma cell line (M10) while failing to lyse other control targets including two allogeneic melanoma cell lines (M11, M12), autologous and allogeneic EBV-B cell lines, and the NK target K562. Blocking experiments indicated that the V β 16 cells have the conventional profile of a HLA class I-restricted CTL. Fig. 3 A shows that cytotoxic activity is inhibited by anti-CD3, anti-CD8, anti-TCR α/β , and anti-V β 16 mAb at the effector cell level and by W6/32 (anti-HLA class I monomorphic mAb) at the target cell level. Furthermore, the presenting HLA class I molecule was characterized with a series of polyclonal antisera corresponding to the HLA phenotype of the patient (HLA-A3, -A10, -B14, -B35). As shown in Fig. 3 B, the anti-HLA-B14 serum was found to substantially reduce cytotoxicity as opposed to the other reagents. Note, that the other previously described clone with antitumor activity char-

acterized in this experimental system was also restricted by HLA-B14, although it expresses a distinct TCR (V β 13, V α 5, or 15, no homology with the V β 16 transcript in the junctional sequence [2]).

Immunohistochemical analysis of the melanoma biopsy with an anti-V β 16 mAb. Because we have previously demonstrated that the great majority of the V β 16 sequences expressed in the tumor correspond to the unique V β 16-LRDSWN-J β 2.1 CTL (1), here we could use the TA-37 mAb to directly visualize the potential antitumor reaction in the lesion. Immunohistochemical experiments (Fig. 4) revealed a substantial V β 16⁺ CTL infiltrate in the peritumoral zone, including the papillary dermis (Fig. 4 B). Furthermore, V β 16⁺ lymphocytes were found directly in the lesion closely opposed to tumor cells (Fig. 4 C). Such a distribution represents a common feature of regressive melanoma lesions (19).

Discussion

The concept of immunosurveillance (20) suggests that transformed cells express tumor-associated antigens that trigger an adaptive immune response aimed at rejection of the developing cancer lesions. This theory has proven extremely difficult to substantiate in the human. Perhaps one of the most relevant arguments is the increase of leukemic relapses in series of patients who have received T cell-depleted bone marrow transplants (21). Regarding solid tumors, it is known that a minority of them, such as melanoma, renal cell carcinoma, or bladder cancer, display so-called “spontaneous” regressions and may respond (22, 23) to various types of immunotherapeutic approaches (e.g., BCG, interferons, IL-2, antiidiotypic antibodies). Yet, these observations constitute only indirect evidence, where actual mechanisms leading to tumor regression are largely unknown.

Malignant melanoma is the most studied model in the field of human tumor immunology. It is well known that the tumors are frequently infiltrated by T lymphocytes, and attempts have been made to correlate the degree of these infiltrations with prognosis (24). In addition, multiple in vitro studies have shown that one can derive from either PBL or TIL IL-2-dependent T cell lines reactive against autologous melanoma cells (reviewed in reference 25). TIL-derived cell lines have even been reinfused to patients in the presence of IL-2 with occasional success (26). Recently, melanoma-associated antigenic peptides, such as those encoded by the MAGE genes, have been characterized and shown to be recognized by autologous CTL (27, 28). Yet, there is still no direct evidence that effective immune responses may occur physiologically in the course of tumor development. Demonstrating the existence of such antitumor reactions and characterizing their modalities is of critical importance in view of future therapeutic immunomodulation strategies aimed at complementing the conventional cancer treatments.

Figure 4. Immunohistological analysis of V β 16⁺ TIL from a regressive melanoma. (A) Hematoxylin-eosin staining of the tumor showing a general view of the tumoral and peritumoral area ($\times 100$). (B and C arrows) Those presented in B and C at higher magnifications ($\times 400$). (B and C) Selected representative sections of the regressive melanoma lesion stained with the anti-V β 16 mAb in the papillary dermis and in the dermal lesion, respectively ($\times 400$). TA-37 mAb (anti-V β 16) was used as 1:50 dilution. A polyclonal biotinylated sheep antibody to mouse Ig and a streptavidin-biotinylated peroxidase complex served as detection system for the primary antibody. 3-Amino-9-ethylcarbazole was used as a substrate for the enzyme.

Our approach has been based on studying a potential local immune response when it is proven clinically and histologically that the tumor "spontaneously" regresses. In a first step (1), it was found that the selected model lesion is indeed infiltrated by T lymphocytes and that some of these TILs represent in situ amplified clonal cell populations. We show here that the major infiltrating clone is a conventional CTL. It has not been established whether this V β 16 CTL is indeed "tumor specific." It is, however, clear that the antitumor activity is selective because both autologous PHA blasts (data not shown) and autologous EBV-B cells (the only available nontumoral cell types from the patient) are not lysed. Further studies aimed at identifying the antigenic peptide(s) that determine(s) the recognition of melanoma cells will clarify this issue. In any case, the present data constitute to our knowledge the first direct evidence that CTL, able to destroy tumor cells, are selected in situ, clonally amplified, and are in a physical situation appropriate to contribute to tumor regression in a clinically relevant (i.e., "spontaneously" regressive) case of a human cancer. These results strongly support the view that the immune system may indeed operate according to the immunosurveillance concept.

Acknowledgments

We thank Dr. M. F. Avril for providing patient materials used in this study, S. Robache for her help in preparing the V β 16 construct, and Dr. F. Faure for advice and discussion. The excellent technical assistance of M. Talbot, I. Billaud, L. Pilatre, and V. Morand is gratefully acknowledged.

This work was supported by Association pour le Recherche contre le Cancer grant 6338. A. Mackensen is a recipient of a fellowship from the Deutsche Forschungsgemeinschaft (Ma 1351/1-1).

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