Analysis of T cell receptor variability in tumor-infiltrating lymphocytes from a human regressive melanoma. Evidence for in situ T cell clonal expansion.

L Ferradini, … , M F Avril, T Hercend

J Clin Invest. 1993;91(3):1183-1190. https://doi.org/10.1172/JCI116278.

Research Article

Malignant melanomas are often infiltrated by T lymphocytes. It is postulated that the presence of tumor-infiltrating lymphocytes (TIL) reflects ongoing immune responses against transformed cells. Such "responses" appear generally inefficient with the potential exception of infrequent clinical situations characterized by spontaneous tumor regression. We have characterized here the molecular structure of the T cell receptor beta chain expressed by TILs in a case of regressive melanoma. Advantage was taken of the PCR technology to study T lymphocytes directly without cell culture. Experimentally validated V beta subfamily specific primers were used to evaluate the V beta usage in TILs and control samples. Our results reveal that clonal T cell populations, precisely defined by their V-D-J junctional sequences, are amplified at the tumor site. The existence of such local antigen-driven selections support the hypothesis that antitumor responses may indeed take place in regressive melanoma.

Analysis of T Cell Receptor Variability in Tumor-infiltrating Lymphocytes from a Human Regressive Melanoma

Evidence for In Situ T Cell Clonal Expansion

Laurent Ferradini, Andreas Mackensen, Catherine Genevée, Jacques Bosq,* Pierre Duvillard,* Marie-Françoise Avril,‡ and Thierry Hercend

*Laboratoire d'Hémato-Immunologie, Institut National de la Santé et de la Recherche Médicale U333; *Département d'Histopathologie and ‡Département de Dermatologie, Institut Gustave Roussy, 94805 Villejuif Cedex, France*

Abstract

Malignant melanomas are often infiltrated by T lymphocytes. It is postulated that the presence of tumor-infiltrating lymphocytes (TIL) reflects ongoing immune responses against transformed cells. Such "responses" appear generally inefficient with the potential exception of unfrequent clinical situations characterized by spontaneous tumor regression. We have characterized here the molecular structure of the T cell receptor β chain expressed by TILs in a case of regressive melanoma. Advantage was taken of the PCR technology to study T lymphocytes directly without cell culture. Experimentally validated Vβ subfamily specific primers were used to evaluate the Vβ usage in TILs and control samples. Our results reveal that clonal T cell populations, precisely defined by their V-D-J junctional sequences, are amplified at the tumor site. The existence of such local antigen-driven selections support the hypothesis that antitumor responses may indeed take place in regressive melanoma. (*J. Clin. Invest.* 1993. 91:1183–1190.)

Key words: melanoma • regression • tumor-infiltrating lymphocytes • T cell receptor • oligoclonality

Introduction

Melanoma is a human cancer where the immune system is thought to play an important role in the control of malignant cell growth. Many in vitro studies have shown the existence of specific immune interactions: cultured T cells derived either from PBL, lymph node, or the site of the tumor were found to display specific MHC-restricted regulatory or effector activities in response to autologous melanoma cells (1–10). Encouraging results have been obtained with ex vivo expanded tumor-infiltrating lymphocytes (TIL)[1] in the presence of IL-2 for the treatment of solid tumors in both animal models (11) and human metastatic melanomas (12, 13). In addition, it is well established that spontaneous regressions (14–17), either partial or complete, occur more frequently in melanoma than in other malignant diseases (14, 18, 19). Such regressions correlate with a high degree of tumor lymphoid infiltration and may well result from an efficient physiological immune response (16). Thus, spontaneously regressing melanoma represents a model of interest to study potential in vivo antitumor responses.

Lymphocytes infiltrating melanoma lesions are mostly $CD3^+$ α/β T lymphocytes (20, 21). It is known that mature α/β T cells specifically recognize antigenic peptides presented by MHC molecules through their heterodimeric surface receptor (TCR) which associates the α and β polypeptides (22). The specific recognition is dependent upon interaction between MHC/peptide complex and the variable region of TCR molecules. During T cell differentiation, unique variable region genes are created by recombination of variable (V), diversity (D) and joining (J) segments for the β locus, and of V and J segments for the α locus. The joining of a random combination of these segments, as well as the pairing of the two chains, generates combinatorial diversity which is greatly increased by imprecise V-D-J (for the β locus) or V-J (for the α locus) joinings (junctional flexibility) and the addition of N-region nucleotides (23). Thus, the expression of unique rearranged TCR gene products determines the specificity of a given T cell.

Since in vitro culture may select and amplify T cell subpopulations with minor representation in vivo, one way to study antitumor immune responses is to analyze directly the molecular structure of TCR polypeptides expressed by TIL. The development of the PCR technology now allows us to perform such experiments. We have used here this method to characterize the local immune response in a case of spontaneously regressive melanoma. Our results show the presence of clonal T cell populations, precisely defined by their V-D-J-C junctional TCR sequences, at the tumor site. Such data strengthen the view that antigen-driven T cell selection may occur locally and contributes to the regression of the tumor.

Methods

Patient characteristics. The patient under study was a 65-yr-old woman suffering from a primary malignant melanoma of the leg, clinically surrounded by an inflammatory area. Histological examination of the tumor revealed the existence of a 4.5-mm thick pigmented mela-

Address correspondence to Laurent Ferradini, M.D., Ph.D., Laboratoire d'Hémato-Immunologie, Institut National de la Santé et de la Recherche Médicale U333, Institut Gustave Roussy, 39 rue Camille Desmoulins, 94805 Villejuif Cedex, France.

Received for publication 8 July 1992 and in revised form 14 October 1992.

1. *Abbreviations used in this paper:* D, diversity; J, joining; TCR, T cell receptor; TIL, tumor-infiltrating lymphocytes; V, variable.

J. Clin. Invest.

0021-9738/93/03/1183/08 $2.00
Volume 91, March 1993, 1183–1190

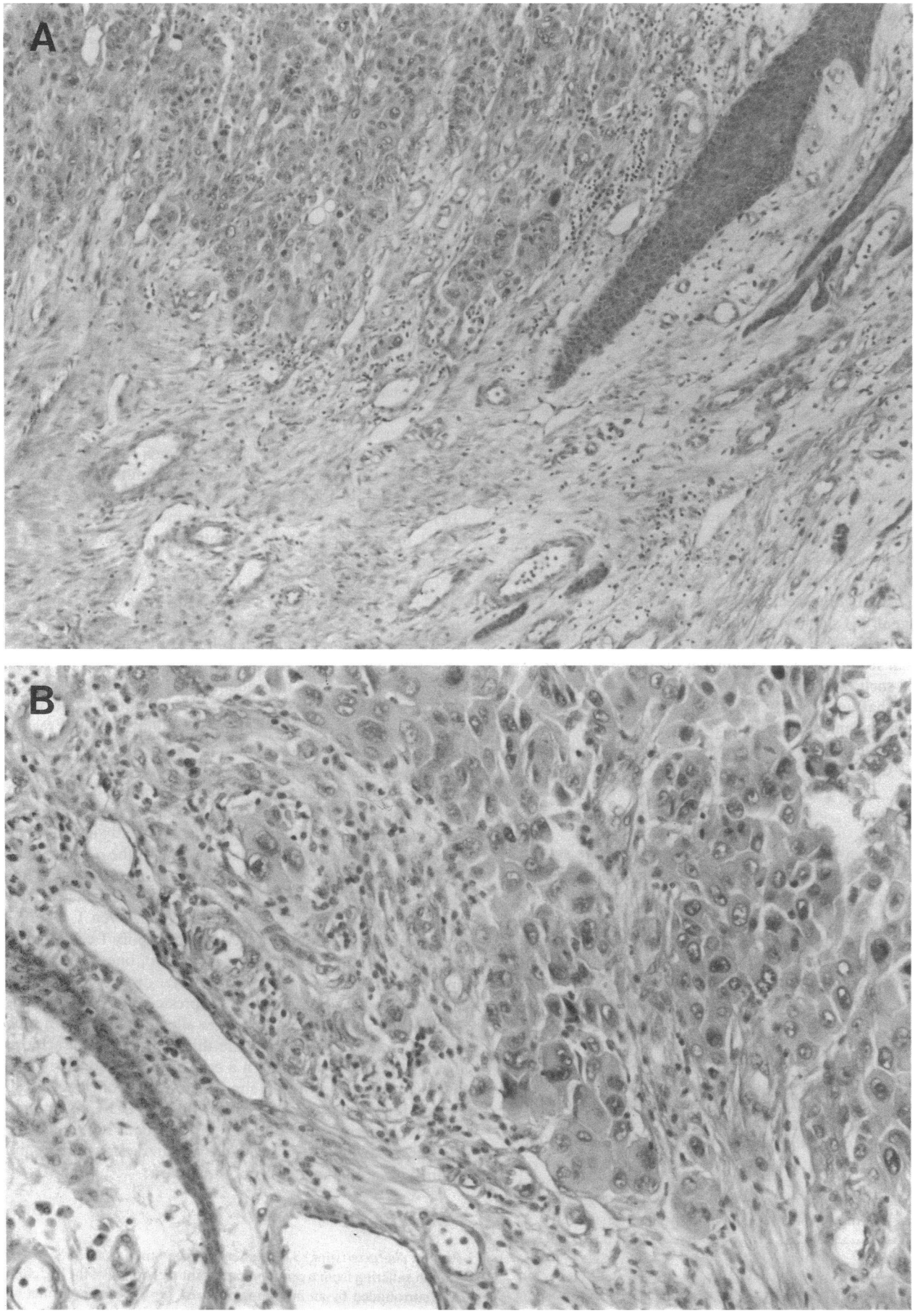

Figure 1.

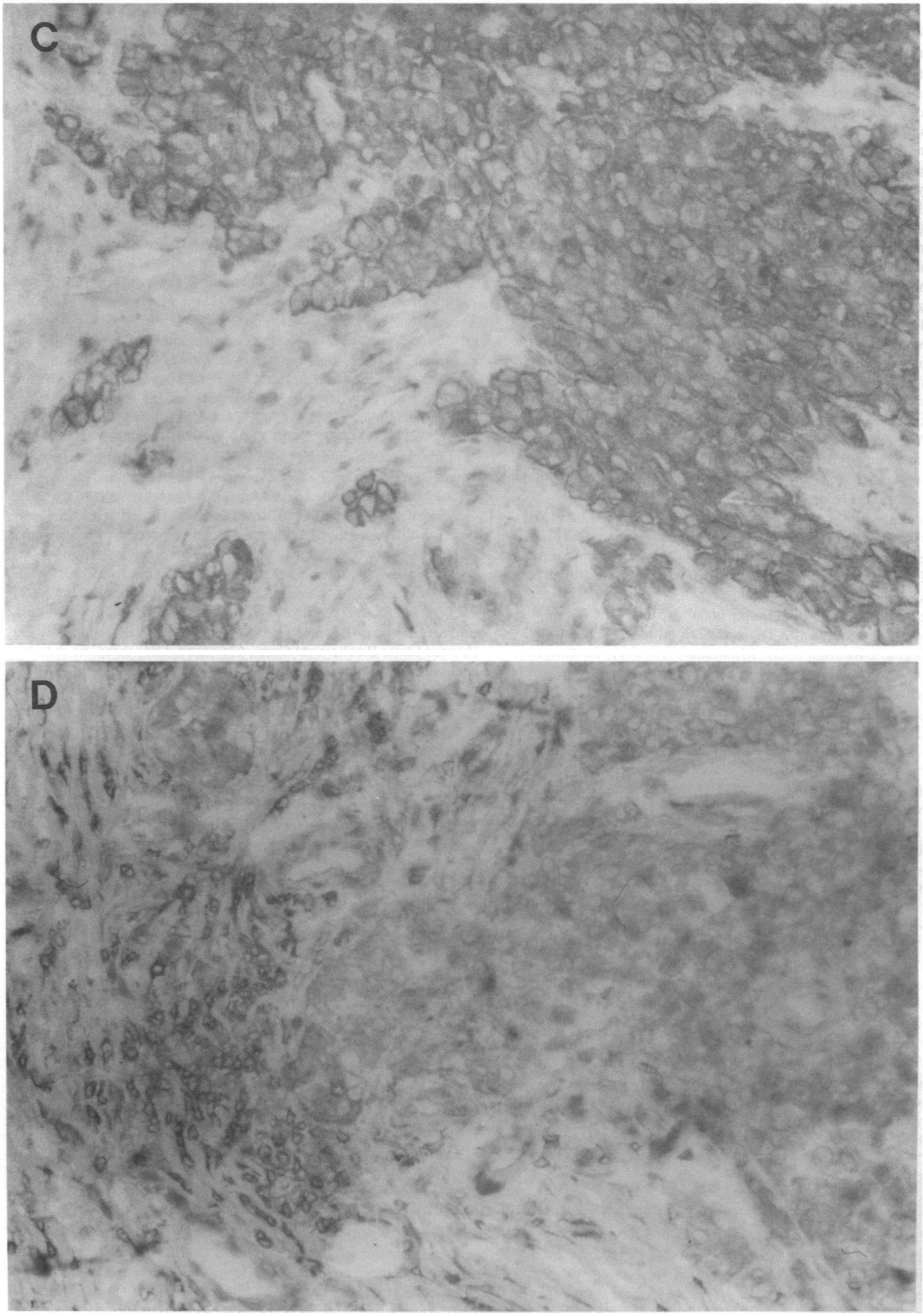

Figure 1 (Continued)

noma (24) with a Clark's level of III (25) composed by atypical globoid cells with high frequency of mitoses. Tumor cells were found to express the protein S100 and to be NSE positive (not shown). Numerous signs of active regression were present in the tumor (Fig. 1, *A* and *B*) and the peritumoral inflammatory skin. Such typical morphologic characteristics of tumor regression (17, 26–28) included dense lymphocytic infiltrates among some degenerating melanoma cells, depigmented epidermis lacking a junctional component, pigmented melanophages revealed by Fontana's coloration, an increased number of capillaries, and foci of fibrosis.

Samples and RNA. After surgical excision, fragments were obtained from tumor, inflammatory, and normal skin of the same patient. Samples were rapidly frozen in liquid nitrogen awaiting RNA extraction. Unstimulated autologous PBL were used as controls. Total RNA was prepared according to the guanidinium isothiocyanate–cesium chloride method (29).

Immunohistochemical staining and mAbs. After surgical excision, a piece of each sample was immersed in isopentane, embedded in OCT compounds, and stored at −80°C until immunohistological staining. Slides were cleaned in 95% alcohol and subbedded in 0.3% gelatin solution containing 0.05% chromium potassium sulfate in distilled water. Cut frozen sections (4-μm thick) were fixed for 10 min with cold acetone and stained with the first mAb. The immunohistological staining was performed using the alkaline phosphatase–anti-alkaline phosphatase complex method (30).

To identify tumor cells, we used the Ep2 mAb (31), (HMW-MAA), that detects the high molecular weight melanoma-associated antigen expressed on a large percentage of human melanoma tumors (32). The determinant recognized by Ep-2 mAb was strongly expressed on nearly all malignant cells (Fig. 1 *C*) but no positive cells could be detected in the peritumoral inflammatory skin. T lymphocytes were studied using anti-CD2, anti-CD3, anti-CD4, and anti-CD8 mAbs (Becton Dickinson & Co., Mountain View, CA). To characterize the TCR, we used βF1 (T Cell Science Co., Cambridge, MA) which recognizes a nonpolymorphic epitope of the β chain expressed by all TCR α/β T cells, and TCRδ1 which reacts with a constant determinant of the TCR δ chain (33) of TCR γ/δ T-cells (kindly provided by M. B. Brenner, Dana-Farber Institute, Boston, MA).

PCR amplifications. First strand cDNA was synthetized from 5 μg of total RNA in a 50-μl final vol at 42°C for 1 h using AMV reverse transcriptase (Stratagene Inc., La Jolla, CA) and a specific Cβ region primer (5′-TATCTGGAGTCATTGAGGGCGGGC-3′).

To study the TCR Vβ repertoire, cDNA/RNA heteroduplex was then submitted to amplification by PCR (34) using a panel of Vβ-specific primers and the same Cβ-specific primer used for cDNA synthesis. The Vβ1-w24 oligonucleotides represent all the known Vβ subfamilies and their specificity has been tested extensively (35). Cβ-Cβ amplifications (190 bp) using a sense Cβ-specific primer (3′-CCCACCC-GAGGTCGCTGTGT-5′) and the antisense Cβ-specific primer were performed as positive controls and to quantify the cDNA following Southern blot analysis. The same amount of specific cDNA from each sample was then used for further amplifications. Negative controls were performed without cDNA. 30 cycles of amplification were performed on a DNA Thermal Cycler (Perkin Elmer Cetus, Emeryville, CA) followed by a 15-min final extension at 72°C. Each cycle consisted of denaturation at 92°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. All experiments were performed at least twice. The amplified products were detected by Southern blot analysis using a Cβ-specific oligonucleotide probe (5′-TCTGCTTCTGATGGC-TCAA-3′).

Autoradiographs were scanned by quantitative densitometry with a dual wavelength chromato-scanner (CS 930; Shimadzu, Kyoto, Japan) providing an absolute value for each autoradiographic spot. Since amplification efficacy is known to vary from a primer pair to another (36), these values did not reflect an absolute image of the repertoire really expressed. For this reason, each Vβ spot was expressed as a percentage of the sum of all Vβ signals detected on the autoradiogram. Since the same Vβ-Cβ primer pair was used in the different samples, intersample comparisons of a given amplified V gene segment could be performed.

For the cloning of Vβ16 sequences, the primer were 5′-GCCTGCA-GAACTGGAGGATTCTGG-3′ in the V region (position 279 from the ATG) and 5′-ACCAGCTCAGCTCCGCGGGGTCGG-3′ in the Cβ region. This latter primer contains an artificial SacII cloning site. The first strand cDNA/RNA heteroduplex was submitted to two rounds of 30 cycles of amplification (34) on a thermocycler (Hybaid, Teddington, UK). Each cycle consisted of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C. The last cycle was followed by a 10-min final extension at 72°C. Between the two rounds of amplification, the material was size purified on a 1% low melting agarose gel. For the amplification of Vβ4 and Vβ19 sequences the primers were 5′-TTCCCATCAGCCGCCCAAACCTAA-3′ for Vβ4 region and 5′-TCC-TCTCACTGTGACATCGGCCCA-3′ for Vβ19 region.

Cloning and sequencing of Vβ16, Vβ4, and Vβ19 transcripts. After ethanol precipitation, half of the second amplification product was digested by SacII and then purified on a 2% agarose gel according to the Geneclean procedure (BIO 101, Inc., La Jolla, CA). The material was ligated into Bluescript SK$^+$ (Stratagene Inc.) digested with SacII and EcoRV and used to transform XL1-blue *Escherichia coli* strains (Stratagene Inc.). The white colonies were screened using a dot blot technique and a third nested Cβ ^{32}P-labeled oligonucleotide (5′-TCTGCTTCTGATGGCTCAA-3′) as a probe. Plasmid DNA was extracted from positive colonies and sequenced with Sequenase 2.0 (United States Biochemical Corp., Cleveland, OH) using the dideoxy chain termination procedure. Note, that to avoid artifacts, all the sequences for a given sample were obtained from at least two independent sets of amplifications and cloning procedure performed on at least two different cDNA.

Contamination. Particular attention was undertaken to avoid sample contamination. All solutions were aliquoted, and aliquots were used only once. Aside from the customary negative controls, a mock sample was processed along with each set of experimental samples during the entire procedure. This control was checked by hybridization with the Cβ probe (data not shown) and ruled out contamination at any step along the way.

Results

Immunohistochemical analysis of melanoma biopsies. Analysis of lymphocyte markers revealed that the tumor was infiltrated by numerous T lymphocytes, expressing the CD2 and CD3 molecules, present both in the stroma and inside tumor cords (Fig. 1 *D*). An equivalent number of CD4 and CD8 positive cells were detected with a majority of CD8 lymphocytes inside the tumor cords. Nearly all of the infiltrating T lymphocytes expressed the TCR α/β as recognized by βF1 (data not shown) while no TCRδ1-positive γ/δ T lymphocytes could be detected.

Figure 1. Histological analysis of tumor cells and TIL from a regressive melanoma. Hematoxylin-eosin staining of the tumor showing signs of spontaneous regression (*A* and *B*) with lymphocytic infiltrate among degenerating melanoma cells (*B*), increased number of capillaries, and foci of fibrosis. Immunochemical staining was performed with the Ep2 mAb detecting the expression of the high molecular weight melanoma-associated antigen on melanoma cells (*C*), and with an anti-CD3 mAb to demonstrate T cell infiltrates in the tumor (*D*). Staining was performed using the alkaline phosphatase anti-alkaline phosphatase complex method (30). Representative fields of the tissue sections examined are shown.

Analysis of the inflammatory peritumoral skin also revealed the presence of α/β T lymphocytes grouped in numerous perivascular foci or dispersed in the derma (data not shown).

Comparative analysis of TCR Vβ gene segment expression. The expression of the 24 Vβ gene subfamilies was analyzed by bispecific PCR performed on the tumor as well as autologous PBL and healthy skin as controls. Quantification of the cDNA samples was performed using Cβ-Cβ amplifications to use the same amount of specific cDNA for Vβ-specific amplifications allowing more accurate comparisons of the Vβ signals. In the case of the healthy skin, little if any T lymphocytes could be identified immunohistologically (data not shown), and Cβ-Cβ as well as Vβ amplifications did not generate signals sufficient to be analyzed by the scanning procedure. Comparing the tumor and autologous PBL, the most striking difference was a 5.7-fold stronger signal for the Vβ16 gene segment in the tumor (Fig. 2). Additional less marked differences (less than twofold) were noted for different Vβ such as Vβ4, Vβ13, or Vβ14 (Fig. 2).

Cloning and sequencing of Vβ16, Vβ4, and Vβ19 cDNA. The quantitative PCR suggested that the Vβ16 gene segment was strongly overexpressed in the tumor. The corresponding transcripts were sequenced to characterize further this potentially in situ expanded pool of Vβ16$^+$ T lymphocytes. A series of control samples were studied in parallel including Vβ16$^+$ cDNA from the patient's healthy skin, autologous PBL, and that from an unrelated healthy donor. Two additional Vβ were selected as controls, one (Vβ4) moderately amplified and the other (Vβ19) lower amplified in the tumor compared to PBL (Fig. 2). To avoid PCR artifacts, sequences were obtained from at least two different sets of amplifications performed on two different cDNA from the same sample. Together, 137 in frame β chain transcripts were characterized. Their complete analysis is summarized in Table I, panel *A*.

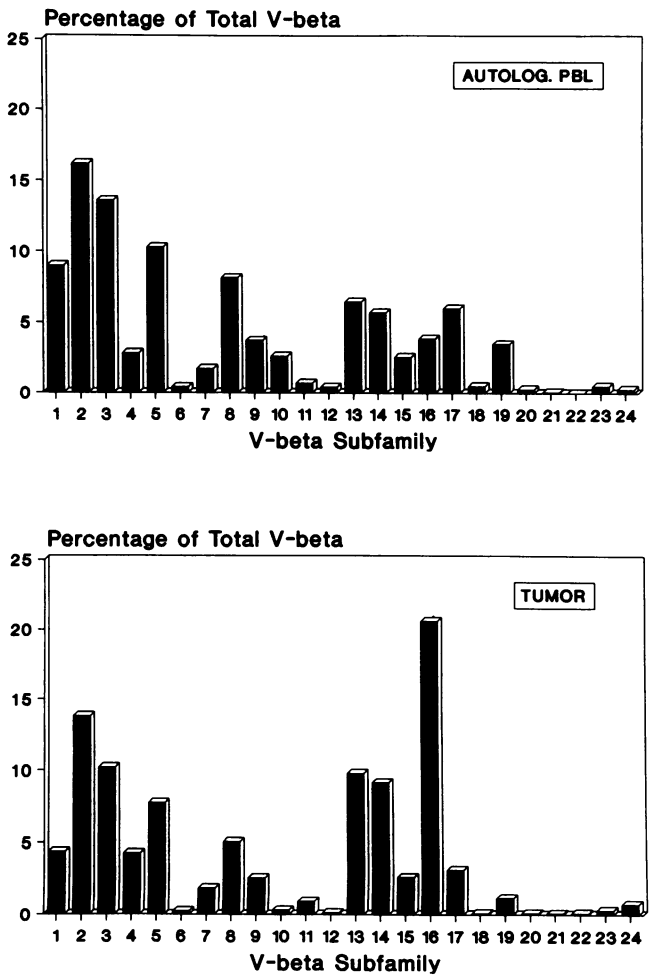

Figure 2. Vβ gene segment usage in human regressive melanoma. Relative Vβ1-w24 subfamily usage in the tumor and the autologous PBL was determined by quantitative PCR and expressed as percentage of the sum of the total Vβ signal obtained after scanning, as indicated in Methods.

The most striking observation was the predominance of a unique cDNA clone in the tumor representing 84% (38:45) of the Vβ16 in frame transcripts and encoding an LRDSWN amino acid junctional sequence (Table I). Another clone accounted for 9% (4:45) of the sequences in the tumor. In contrast, Vβ16 β-transcripts from autologous control PBL did not occur more than two times reflecting a regular polyclonal distribution (Table I). A similar distribution was found when sequencing Vβ16 β transcripts from unrelated allogeneic PBL (data not shown). Note that the predominant clone with the LRDSWN amino acid junctional sequence, present in the tumor, was not identified among autologous PBL Vβ16 transcripts. No Vβ16 transcript could be amplified and cloned from patient's healthy skin.

Analysis of the Vβ4 in frame transcripts revealed that a unique cDNA clone, encoding a QTSVVV junctional sequence, represented 47% (14:30) of the Vβ4 transcripts in the tumor but was not identified in autologous PBL. Another cDNA clone, encoding for an ENGRE junctional sequence, was found in 3:30 sequences (10%) in the tumor. The Vβ4$^+$ cDNA clones from autologous PBL were polyclonal, in that none of the sequences examined occurred more than two times (Table I, panel *A*). Analysis of the Vβ19 sequences revealed that 2 Vβ19 clones occurred 5:18 and 3:18 times in the tumor whereas no sequences occurred more than two times in autologous PBL.

Sequencing Vβ16, Vβ4, and Vβ19 cDNA from the inflammatory peritumoral skin. Histological examination of the inflammatory peritumoral skin has revealed signs of regression including lymphocyte infiltration, lack of normal junctional melanocytes, and fibrosis (data not shown). No melanoma cells were detectable with the Ep-2 mAb specific for the HMW-MAA (31, 32) suggesting that tumor cells had been eradicated from this area. Therefore, it was of interest to assess whether the clones identified in the tumor were also present in the inflammatory peritumoral skin. As shown in Table I, panel *B*, the LRDSWN predominant Vβ16 cDNA clone represented 100% (28:28) of the Vβ16 in frame transcripts studied in this area. Similarly, when looking at the Vβ4 gene segment, the two clones sharing the QTSVVV and the ENGRE junctional regions could be identified in 26% and 16% of the transcripts studied, respectively. On the other hand, several Vβ4$^+$ cDNA clones were present only in the inflammatory peritumoral skin and not in the tumor. One of them, sharing a SRTGM junctional sequence represented 26% (5:19) of the transcripts in the inflammatory skin, and was not found elsewhere. In contrast, Vβ19 transcripts were different from those present in the tumor and were not found more than two times.

Table I. Analysis of TCR β Chain Transcripts Expressed in the Tumor, Autologous PBL, and Inflammatory Peritumoral Skin

Sample	No. of sequences	Sequences occurring more than two times: No. of occurrences	Junctional sequence: Vβ	NDβN	Jβ	Sequences occurring one or two times: No. of occurrences	Jβ1 123456	Jβ2 1234567
		%				%		
A								
Tumor								
Vβ16	45	38 (84)	CASS	LRDSWN	YEQF (2.1)	3 (7)	000000	0100002
		4 (9)	CASS	QGVGRK	AKNIQYF (2.4)			
Vβ4	30	14 (47)	CSVE	QTSVVV	EQFF (2.1)	13 (43)	220000	1030005
		3 (10)	CSVE	ENGRE	YNEQFF (2.1)			
Vβ19	18	5 (28)	CASS	LLQGP	NYGYTF (1.2)	10 (55)	010021	0130110
		3 (17)	CASS	IQSGG	YEQYR (2.7)			
PBL								
Vβ16	16	0 (0)				16 (100)	320300	2110112
Vβ4	15	0 (0)				15 (100)	040000	6010103
Vβ19	13	0 (0)				13 (100)	200010	3101302
B								
Inflammatory peritumoral skin								
Vβ16	28	28 (100)	CASS	LRDSWN	YEQF (2.1)	0 (0)	000000	0000000
Vβ4	19	5 (26)	CSVE	QTSVVV	EQFF (2.1)	6 (32)	000000	4020000
		5 (26)	CS	SRTGM	TEAFF (1.1)			
		3 (16)	CSVE	ENGRE	YNEQFF (2.1)			
Vβ19	11	0 (0)				12 (100)	240200	0210100

All PCR-generated Vβ16, Vβ4, and Vβ19-TCR fragments were cloned into SacII-EcoRV pBS-SK$^+$ vector and sequenced as indicated in Methods. The encoded amino acid sequences of the junctional regions of clones occuring more than two times are shown according to the one letter code.

Discussion

Regressive melanoma represents a privileged situation where the often abundant T lymphocytes closely apposed to regressing nests of tumor cells (15–18) may well display an efficient in vivo immune response. We have studied here a clinically and pathologically representative case where well recognized histological criteria of tumor regression (17, 26–28) were present (see Fig. 1, *A* and *B*). Immunohistological analysis demonstrated the presence of numerous α/β T cells in the tumor. Since monoclonal antibodies directed against each of the distinct V region of the TCR are not yet available, analysis of TCR transcripts represents an acceptable alternative to study the T cell repertoire. We have analyzed TCR β chain transcripts because, similar to immunoglobulin loci, allelic exclusion is known to operate efficiently at the RNA level for the TCR β locus with only one in frame transcript expressed by a given T cell (37). Note that this may not be the case for the TCR α loci where two in frame transcripts have been shown to be frequently expressed (38–40).

The TCR Vβ usage of TIL and controls was first evaluated by PCR using Vβ subfamily specific oligonucleotides that have been experimentally validated in previous studies (35). Vβ-specific amplifications revealed one major difference between the tumor and the autologous PBL, namely, an overrepresentation of the Vβ16 gene segment. Sequences were then performed to analyze the V-D-J-C junctional regions of the Vβ16 transcripts. The results showed that 84% of the Vβ16 transcripts analyzed in the tumor corresponded to the same cDNA clone while all were different in autologous PBL. Together, these data strongly support the view that clonal expansion of a Vβ16$^+$ T cell occurred locally. Studying Vβ4 transcripts, which were moderately amplified in the tumor, we observed that one cDNA clone represented almost 47% of the transcripts in the tumor while all transcripts in PBL were different. Analyzing Vβ19 transcripts, which were less represented in the tumor (Fig. 2), we found that two clones accounted for 28 and 17% of the transcripts while the distribution was polyclonal in PBL.

The approach used here, namely, Vβ-specific amplifications, allowed us to select the Vβ16 subfamily for subsequent sequence experiments and to identify a Vβ16 clonal expansion in the tumor. Analysis of the control Vβs indicated that clonal expansion of other T cells had occurred locally: it shows that strictly quantitative analyses may be misleading and outlines the importance of sequence analysis in fine studies on T cell repertoire. However, such experiments are presently extremely tedious and it is clear that novel more rapid (automated) methods have to be developed to allow extensive characterization of the T cell repertoire in immunopathological situations.

A limited number of comparable studies have been performed directly in situ or on freshly isolated TIL using either Southern blot analysis of TCR β chain rearrangements (41), TCR V region specific PCR alone (42), or anchored PCR (43). Nitta et al. (42) have reported the preferential usage of

the Vα7 gene segment in uveal primary melanoma with no preferential usage of any Vβ gene segments. However, without sequence analysis, one cannot conclude that in situ clonal T cell expansion is present. We have recently performed sequence analysis using anchored PCR in metastatic melanoma and did not observe preferential Vα or Vβ gene segment usage (43). In vitro studies have reported oligoclonality of IL-2-expanded TIL in melanoma (44–46). However, the relevance of the latter data is difficult to appreciate since cultures, particularly long term, may select T cell subpopulations poorly represented in vivo.

To our knowledge, the present observation based on sequence analysis of TCR transcripts constitutes an original demonstration that clonally expanded T cell populations may be present at the tumor site in a human cancer. The fact that the same Vβ16 and Vβ4 clones were identified in the inflammatory peritumoral skin, where more advanced histological signs of regression were observed, strengthens both the technical validity and the biological relevance of these findings. The demonstration of in situ clonal T cell expansion strongly suggests that antigen driven selection occurs at the tumor site. The relationship between the regression phenomena and amplification of clonal T cells have to be confirmed by functional studies.

Acknowledgments

We thank Professor Prade and Drs. F. Praz and F. Triebel for critical review of the manuscript and helpful discussion. We thank Sophie Viel, Delphine Delorme, and Monique Talbot for their dedicated technical help.

This work was supported in part by ADRC grants No. 2110 and 6338. A. Mackensen is a recipient of a fellowship from the Deutsche Forschungsgemeinschaft (Ma 1351/1-1).

References

1. Anichini, A., G. Fossati, and G. Parmiani. 1985. Clonal analysis of cytotoxic T-lymphocyte response to autologous human metastatic melanoma. *Int. J. Cancer.* 35:683–689.

2. Mesler Muul, L., P. J. Spiess, E. P. Director, and S. A. Rosenberg. 1987. Identification of specific cytolytic immune responses against autologous tumor in humans bearing malignant melanoma. *J. Immunol.* 138:989–995.

3. Herin, M., C. Lemoine, P. Weynants, F. Vessiere, A. Van Pel, A. Knuth, R. Devos, and T. Boon. 1987. Production of stable cytolytic T-cell clones directed against autologous human melanoma. *Int. J. Cancer.* 39:390–396.

4. Itoh, K., C. D. Platsoucas, and C. M. Balch. 1988. Autologous tumor-specific cytotoxic T lymphocytes in the infiltrate of human metastatic melanomas: activation by interleukin-2 and autologous tumor cells, and involvement of the T cell receptor. *J. Exp. Med.* 168:1419–1441.

5. Wolfel, T., E. Klehmann, C. Muller, K. H. Schutt, K. H. Meyer Zum Buschenfelde, and A. Knuth. 1989. Lysis of human melanoma cells by autologous cytolytic T cell clones. *J. Exp. Med.* 170:797–810.

6. Mukherji, B., A. Guha, N. G. Chakraborty, M. Sivanandham, A. L. Nashed, J. R. Sporn, and M. T. Ergin. 1989. Clonal analysis of cytotoxic and regulatory T cell responses against human melanoma. *J. Exp. Med.* 169:1961–1976.

7. Chakraborty, N. G., D. R. Twardzik, M. Sivanandham, M. T. Ergin, K. E. Hellstrom, and B. Mukherji. 1990. Autologous melanoma-induced activation of regulatory T cells that suppress cytotoxic response. *J. Immunol.* 145:2359–2364.

8. Topalian, S. L., D. Solomon, and S. A. Rosenberg. 1989. Tumor-specific cytolysis by lymphocytes infiltrating human melanomas. *J. Immunol.* 142:3714–3725.

9. Knuth, A., T. Wolfel, E. Klehmann, T. Boon, and K. H. Meyer zum Buschenfelde. 1989. Cytolytic T-cell clones against an autologous human melanoma: specificity study and definition of three antigens by immunoselection. *Proc. Natl. Acad. Sci. USA.* 86:2804–2808.

10. Darrow, T. L., C. L. Slingluff, and H. F. Seigler. 1989. The role of HLA class I antigens in recognition of melanoma cells by tumor-specific cytotoxic T lymphocytes. Evidence for shared tumor antigens. *J. Immunol.* 142:3329–3335.

11. Rosenberg, S. A., P. Spiess, and R. Lafreniere. 1986. A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes. *Science (Wash. DC).* 233:1318–1321.

12. Rosenberg, S. A., B. S. Packard, P. M. Aebersold, D. Salomon, S. L. Topalian, S. T. Toy, P. Simon, M. T. Lotze, J. C. Yang, C. A. Seipp, et al. 1988. Use of tumor infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. *N. Engl. J. Med.* 319:1676–1680.

13. Topalian, S. L., D. Solomon, F. P. Avis, A. E. Chang, D. L. Freerksen, W. M. Linehan, M. T. Lotze, C. N. Robertson, C. A. Seipp, P. Simon, C. G. Simpson, and S. A. Rosenberg. 1988. Immunotherapy of patients with advanced cancer using tumor-infiltrating lymphocytes and recombinant interleukin-2: a pilot study. *J. Clin. Oncol.* 6:839–853.

14. Everson, T. C., and W. H. Cole. 1966. Spontaneous regression of cancer. W. B. Saunders Co., Philadelphia. 164–220.

15. McGovern, V. J. 1975. Spontaneous regression of melanoma. *Pathology.* 7:91–99.

16. Nathanson, L. 1976. Spontaneous regression of malignant melanoma: a review of the literature on incidence, clinical features, and possible mechanisms. Conference on spontaneous regression of cancer. *Natl. Cancer Inst. Monogr.* 44:67–76.

17. Bodurtha, A. J. 1979. Spontaneous regression of malignant melanoma. *In* Human Malignant Melanoma. W. H. Clark, L. I. Goldman, and J. M. Mastrangelo, editors. Grune and Stratton, New York. 227–241.

18. McGovern, V. J. 1972. Melanoma-growth patterns, multiplicity and regression. *In* Melanoma and Skin Cancer. Proceedings of the international cancer conferences. Government printer, V. C. N. Blight, Sydney. 95–106.

19. Little, J. H. 1972. Histology and prognosis in cutaneous malignant melanoma. *In* Melanoma and Skin Cancer. Proceedings of the international cancer conferences. Government printer, V. C. N. Blight, Sydney. 107–120.

20. Poppema, S., E. B. Brocker, L. De Leij, D. Terbrack, T. Visscher, A. Ter Haar, E. Macher, T. H. Thé, and C. Sorg. 1983. In situ analysis of the mononuclear cell infiltrate in primary malignant melanoma of the skin. *Clin. Exp. Immunol.* 51:77–82.

21. Cardi, G., M. J. Mastrangelo, and D. Berd. 1989. Depletion of T-cells with the CD4+CD45R+ phenotype in lymphocytes that infiltrate subcutaneous metastases of human melanoma. *Cancer Res.* 49:6562–6565.

22. Meuer, S. C., K. A. Fitzgerald, R. E. Hussey, J. C. Hodgdon, S. F. Schlossman, and E. L. Reinherz. 1983. Clonotypic structures involved in antigen-specific human T cell function. *J. Exp. Med.* 157:705–719.

23. Davis, M. M., and P. J. Bjorkman. 1988. T-cell antigen receptor genes and T-cell recognition. *Nature (Lond.).* 334:395–402.

24. Breslow, A. 1970. Thickness, cross-sectional areas and depth of invasion in the prognosis of cutaneous melanoma. *Ann. Surg.* 172:902–908.

25. Clark, W. H. 1967. A classification of malignant melanoma in man correlated with histogenesis and biologic behavior. *In* Advances in Biology of the Skin. Vol. 8. The Pigmentary System. W. Montagna and F. Hu, editors. Pergamon Press, London. 621–632.

26. Smith, J. L., and J. S. Stehlin. 1965. Spontaneous regression of primary malignant melanomas with regional metastasis. *Cancer (Phila.).* 18:1399–1415.

27. McGovern, V. J., H. M. Shaw, and G. W. Milton. 1983. Prognosis in patients with thin malignant melanoma: influence of regression. *Histopathology (Oxf.).* 7:673–680.

28. Ronan, S. G., A. M. Eng, H. A. Briele, N. N. Shioura, and T. K. Das Gupta. 1987. Thin malignant melanomas with regression and metastases. *Arch. Dermatol.* 123:1326–1330.

29. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5299.

30. Cordell, J. L., B. Falini, W. N. Erber, A. K. Ghosh, Z. Abdulaziz, S. Macdonald, K. A. F. Pulford, H. Stein, and D. Y. Mason. 1984. Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase. *J. Histochem. Cytochem.* 32:219–229.

31. Giacomini, P., O. Segatto, and P. G. Natali. 1987. Multiple epitope recognition: an approach to improved radioimmunodetection of tumor-associated antigens. *Int. J. Cancer.* 39:729–736.

32. Natali, P. G., K. Imai, B. S. Wilson, A. Bigotti, R. Cavaliere, M. A. Pellegrino, and S. Ferrone. 1981. Structural properties and tissue distribution of the antigen recognized by the monoclonal antibody 653.40s to human melanoma cells. *J. Natl. Cancer Inst. (Bethesda).* 76:591–601.

33. Band, H., F. Hochstenbach, J. McLean, S. Hata, M. S. Krangel, and M. B. Brenner. 1987. Immunochemical proof that a novel rearranging gene encodes the T cell receptor delta subunit. *Science (Wash. DC).* 238:682–685.

34. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science (Wash. DC).* 239:487–491.

35. Genevée, C., A. Diu, J. Nierat, A. Caignard, P.-Y. Dietrich, L. Ferradini, S. Roman-Roman, F. Triebel, and T. Hercend. 1992. An experimentally validated panel of subfamily-specific oligonucleotide primers (Vα1-w29/Vβ1-w24)

for the study of human T-cell receptor V gene segment usage by polymerase chain reaction. *Eur. J. Immunol.* 22:1261–1269.

36. Wang, A. M., M. V. Doyle, and D. F. Mark. 1989. Quantitation of mRNA by the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA.* 86:9717–9721.

37. Uematsu, Y., S. Ryser, Z. Dembic, P. Borgulya, P. Krimpenfort, A. Berns, H. Von Boehmer, and M. Steinmetz. 1988. In transgenic mice the introduced functional T cell receptor beta gene prevents expression of endogenous beta genes. *Cell.* 52:831–841.

38. Furutani, M., Y. Yanagi, I. Fujisawa, T. Nakayama, H. Kishimoto, K. Kuida, Y. Asano, and T. Tada. 1989. Post-transcriptional allelic exclusion of two functionally rearranged T cell receptor α genes. *Int. Immunol.* 1:281–288.

39. Blütmann, H., P. Kisielow, Y. Uematsu, M. Malissen, P. Krimpenfort, A. Berns, H. Von Boehmer, and M. Steinmetz. 1988. T-cell specific deletion of T-cell receptor transgenes allows functional rearrangement of endogenous α- and β-genes. *Nature (Lond.).* 334:156–159.

40. Couez, D., M. Malissen, M. Buferne, A. M. Schmitt-Verhulst, and B. Malissen. 1991. Each of the two productive T cell receptor α-gene rearrangements found in both the A10 and BM3.3 T cell clones give rise to an α chain which can contribute to the constitution of a surface-expressed $\alpha\beta$ dimer. *Int. Immunol.* 3:719–729.

41. Yoshino, I., T. Yano, Y. Yoshikai, M. Murata, K. Sugimachi, G. Kimura, and K. Nomoto. 1991. Oligoclonal T lymphocytes infiltrating human lung cancer tissues. *Int. J. Cancer.* 47:654–658.

42. Nitta, T., J. R. Oksenberg, N. A. Rao, and L. Steinman. 1990. Predominant expression of T cell receptor Valpha7 in tumor-infiltrating lymphocytes of uveal melanoma. *Science (Wash. DC).* 249:672–674.

43. Ferradini, L., S. Roman-Roman, J. Azocar, M. F. Avril, S. Viel, F. Triebel, and T. Hercend. 1992. Analysis of T-cell receptor α/β variability in lymphocytes infiltrating a melanoma metastasis. *Cancer Res.* 52:4649–4654.

44. Belldegrun, A., A. Kasid, M. Uppenkamp, S. L. Topalian, and S. A. Rosenberg. 1989. Human tumor infiltrating lymphocytes. *J. Immunol.* 42:4520–4526.

45. Gervois, N., F. Heuze, E. Diez, and F. Jotereau. 1990. Selective expansion of a specific anti-tumor $CD8^+$ cytotoxic T lymphocyte clone in the bulk culture of tumor-infiltrating lymphocytes from a melanoma patient: cytotoxic activity and T cell receptor gene rearrangements. *Eur. J. Immunol.* 20:825–831.

46. Morita, T., M. A. Salmeron, R. P. Moser, M. I. Ross, and K. Itoh. 1992. Oligoclonal expansion of $V\beta8^+$ cells in interleukin-2-activated T cells residing in subcutaneous metastatic melanoma. *Clin. Exp. Metastasis.* 10:69–76.