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Cytotoxic T-lymphocyte clones from different patients display limited T-cell-receptor variable-region gene usage in HLA-A2-restricted recognition of the melanoma antigen Melan-A/MART-1

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ABSTRACT **To determine whether T-cell-receptor (TCR) usage by T cells recognizing a defined human tumor antigen in the context of the same HLA molecule is conserved, we analyzed the TCR diversity of autologous HLA-A2-restricted cytotoxic T-lymphocyte (CTL) clones derived from five patients with metastatic melanoma and specific for the common melanoma antigen Melan-A/MART-1. These clones were first identified among HLA-A2-restricted anti-melanoma CTL clones by their ability to specifically release tumor necrosis factor in response to HLA-A2.1$^+$ COS-7 cells expressing this tumor antigen. A PCR with variable (V)-region gene subfamily-specific primers was performed on cDNA from each clone followed by DNA sequencing. TCRAV2S1 was the predominant α-chain V region, being transcribed in 6 out of 9 Melan-A/MART-1-specific CTL clones obtained from the five patients. β-chain V-region usage was also restricted, with either TCRBV14 or TCRBV7 expressed by all but one clone. In addition, a conserved TCRAV2S1/TCRBV14 combination was expressed in four CTL clones from three patients. None of these V-region genes was found in a group of four HLA-A2-restricted CTL clones recognizing different antigens (e.g., tyrosinase) on the autologous tumor. TCR joining regions were heterogeneous, although conserved structural features were observed in the complementarity-determining region 3 sequences. These results indicate that a selective repertoire of TCR genes is used in anti-melanoma responses when the response is narrowed to major histocompatibility complex-restricted antigen-specific interactions.**

The analysis of T-cell receptors (TCRs) expressed by T cells infiltrating human melanomas has been the subject of several investigations (1–9). In uveal melanoma (1), expression of one defined TCRA variable (V)-region gene family was observed for the majority of these tumors. Restricted use of TCRV genes has also been shown in some but not other cases of individual lesions in both primary and metastatic melanoma, often as a result of an oligoclonal expansion (2–4, 6). These studies, however, did not provide information on the functional significance of T cells expressing given V-region genes either on the target antigens or on the restricting HLA molecule involved. Therefore, only TCR analysis of T-cell clones with defined antigenic specificity can provide insight on the TCR repertoire involved in the recognition of tumor antigens (5, 7–9). By using this approach, we have recently shown that the TCR α- and β-chain repertoire of HLA-A2-restricted cytotoxic T-lymphocyte (CTL) clones directed to melanocyte-lineage-specific antigens included a few TCR combinations that correlated with different specificity patterns of the CTL clones (5). The molecular nature of the antigen(s) recognized was, however, unknown.

A number of genes encoding melanoma antigens recognized by autologous T cells have been cloned (10–15). In agreement with the observation that HLA-A2 patients have CTL clones reacting with both the autologous melanoma and HLA-A2-matched melanocytes (16), all genes that encode shared HLA-A2-restricted antigens identified so far are expressed in melanocytes and melanoma (11–15). Two of these genes code for tyrosinase, a key enzyme in melanin biosynthesis, and gp100, a protein component of the same biosynthetic pathway (11–13). A third gene encodes a protein of still unknown function and has been independently cloned by Coulie *et al.* (14) and Kawakami *et al.* (15). We will refer to the antigen encoded by this gene as Melan-A/MART-1, thus conserving both original designations.

The identification of genes coding for shared melanoma antigens allows us to study the extent of TCR diversity among T cells that recognize a known human tumor antigen presented by a specific major histocompatibility complex class I molecule. Here, we have analyzed the TCR structure of CTL clones** isolated from different HLA-A2$^+$ melanoma patients and directed to Melan-A/MART-1. The results indicate that limited TCR V regions are used in different individuals for recognition of Melan-A/MART-1 in the context of HLA-A2.

MATERIALS AND METHODS

Neoplastic Cells. Melanoma 8959 (HLA-A2, A; BW55, B; CW3, C; DRW11, DR; DQW7, DQ), isolated from a lymph node metastasis, was established *in vitro* as described (16). The characterization and culture conditions of melanomas 9742, LB39, MZ2-Mel, and SK29-1.2 (an HLA-A2-negative variant of SK29) and of normal human melanocytes, kindly provided by Meenhard Herlyn (Wistar Institute, Philadelphia), have been reported (14, 16–18).

Generation of CTL Clones and Cytotoxicity Assay. Two mixed-lymphocyte tumor cultures (1A or 2A) of peripheral blood lymphocytes (PBLs) and melanoma cells isolated from patient 8959 were prepared as described (5). Cloning of both cultures was carried out by seeding 5, 1, or 0.5 cells per well in

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Abbreviations: TCR, T-cell receptor; V, variable; CTL, cytotoxic T lymphocyte; PBL, peripheral blood lymphocyte; TNF, tumor necrosis factor; J, joining; D, diversity; C, constant; CDR, complementarity-determining region.

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**The sequences reported in this paper have been deposited in the GenBank data base (accession nos. X83772–X83789).

round-bottom 96-well plates (Costar 3596) in RPMI medium 1640 (BioWhittaker) supplemented with 10% (vol/vol) fetal calf serum (Biological Industries, Beth Haemek, Israel), interleukin 2 (EuroCetus, Amsterdam; 300 international units/ml), irradiated (5000 rad; 1 rad = 0.01 Gy) allogeneic PBLs (5×10^4 cells per well), and purified phytohemagglutinin at 1 μg/ml (HA16, MUREX, Temple Hill Dartford, England). Growing clones were transferred into 24-well plates and restimulated as described above. The probability of clonality was calculated by Poisson statistics (19) and only clones with a *P* value for clonality >0.95 were further analyzed. CTL clones were tested for cytotoxicity in a 4-h ^{51}Cr-release assay (16). Inhibition of lysis by monoclonal antibody to CD3 (OKT3, American Type Culture Collection) and to HLA-A2 (CR11.351), kindly provided by C. Russo (Cornell University, New York), was performed as described (16). The derivation and maintenance of CTL clones A81, 1/95, 10/196, 11/33, 2/9, and A42 have been reported (5, 14, 15, 17).

RNA Extraction, cDNA Synthesis, and PCR. Total RNA was prepared from CTL clones by using RNAzol·B (Cinna/Biotecx, Friendswood, TX) and first-strand cDNA was synthesized with oligo(dT) and reverse transcriptase (Superscript, GIBCO/BRL). PCR was carried out by amplification with primers complementary to TCR V and constant (C)-region sequences (20) in a 25-μl reaction mixture containing 0.5 μl of cDNA, all four dNTPs (each at 200 μM), 1 μM of each primer, and 0.625 unit of *Taq* polymerase (Ampli Taq, Perkin–Elmer/Cetus) on a DNA thermal cycler (model 9600 GeneAmp PCR system, Perkin–Elmer/Cetus). Amplification was performed for 30 cycles, each consisting of 1 min at 95°C, 30 sec at 60°C, and 1 min at 72°C. All oligonucleotides were prepared on a model 380A DNA synthesizer (Applied Biosystems).

Cloning of PCR-Amplified Products and Sequencing Reactions. PCR products were cloned into the T/A vector PCR II (TA cloning kit, Invitrogen) and sequenced with Sequenase 2.0 (United States Biochemicals). Nucleotide sequences were compared to gene data bank entries and to available published TCR sequences (21–30). TCRV gene segments were classified according to family designations outlined by Wilson *et al.* (31). We have adopted the TCR nomenclature proposed by the International Union of Immunological Societies (32).

Antigen Recognition by CTL Clones. Transient transfections were performed by the DEAE-dextran/cloroquine method (14). Briefly, 10^4 COS-7 cells were seeded in wells of a 96-well flat-bottom plate 24 h before transfection with 100 ng of pcDNAI/Amp (Invitrogen) encoding HLA-A2.1 (14) and 100 ng of pcDNAI/Amp encoding each of the different tumor antigens (10, 11, 14). Transfected COS-7 cells were incubated for 24 h at 37°C. The medium was then discarded and 10^3 CTLs were added in 100 μl of Iscove medium (GIBCO/BRL) supplemented with 10% (vol/vol) human serum and interleukin 2 (60 international units/ml). After 24 h, the supernatant was collected and its tumor necrosis factor (TNF) content was determined by its cytolytic effect on WEHI-164 clone 13 as described (10).

RESULTS

TCRV Gene Usage and Melanoma Antigens Recognized by HLA-A2-Restricted CTL Clones of Patients 8959 and 9742. We have shown (5) specific recognition of melanocyte-related differentiation antigen(s) in the context of HLA-A2 molecule by CTL clones expressing particular TCR combinations and derived from a patient with metastatic melanoma (patient 9742). To understand whether particular clonotypes could be frequently involved in the T-cell response to melanoma, additional HLA-A2-restricted CTL clones, isolated from PBLs of patient 8959, were selected for TCR analysis. All these clones displayed lytic activity on the autologous melanoma that was inhibited by anti-CD3 and anti-HLA-A2 monoclonal antibodies (Table 1). In addition, CTL clones 1A9/4, 1A77, 2A9, 2A13, 2A27, and 2A37, but not 2A22 or 2A121, lysed HLA-matched melanocytes in an HLA-A2-restricted fashion. Table 1 shows that CTL clones that recognized both melanoma and melanocytes expressed TCRAV2/TCRBV14 (2A9, 2A13, and 2A27), VA2/VB4 (1A77), and VA21/VB5 (2A37). For CTL clone 1A9/4, only the V region used in its β chain (VB6) could be determined. Both clones that lysed the autologous melanoma but spared HLA-A2-matched melanocytes used a VA3/VB8 (Table 1). VA2/VB14 was the predominant combination in PBL clones derived from patient 9742 recognizing both melanoma and melanocytes in an HLA-A2-restricted fashion (5). To determine whether a correlation exists between TCRV gene usage and fine specificity, antigen recognition for all CTL clones from patient 8959 and a VA2/VB14 CTL clone from patient 9742 (A81) was determined. CTL clones were tested for their ability to produce TNF in response to HLA-A2$^+$ melanoma cells or to COS-7 cells transfected with expression vectors encoding different melanoma antigens and HLA-A2.1 (Table 2). Table 2 shows that all CTL clones produced TNF when stimulated by the autologous melanoma. HLA-A2.1$^+$ COS-7 cells expressing Melan-A/MART-1 reproducibly stimulated TNF release by five out of nine CTL clones (Table 2). The same CTL clones could also be stimulated by HLA-A2$^+$ allogeneic melanomas and, when tested, by MZ-2 melanoma stably transfected with HLA-A2.1 and Melan-A/MART-1 genes. Less than 5 pg of TNF per ml was produced when the same CTL clones were assayed on SK29.1.22 melanoma (HLA-A2$^-$) or on COS-7 cells expressing only HLA-A2.1 or Melan-A/MART-1. Tyrosinase reconstituted recognition by CTL clone 2A37 whereas none of the transfected genes could stimulate a response by clone 1A9/4. CTL clones 2A22 and

Table 1. Cytolytic activity and TCRV gene expression of autologous anti-melanoma CTL clones from patient 8959

	% lysis of melanoma 8959			% lysis of FM927 melanocytes		TCR usage	
CTL clone	No mAb	αCD3	αHLA-A2	No mAb	αHLA-A2	TCRAV	TCRBV
1A9/4	49	6	26	55	ND	ND	6
1A77	24	5	4	22	4	2	4
2A9	51	3	27	50	6	2	14
2A13	52	11	3	51	ND	2	14
2A27	39	8	21	30	ND	2	14
2A37	25	7	16	22	1	21	5
2A22	32	8	17	1	2	3	8
2A121	24	3	15	0	2	3	8

CTL clones were tested for lysis of the autologous tumor and of HLA-A2 allogeneic FM927 melanocytes at an effector/target ratio of 10:1 in a 4-h ^{51}Cr-release assay (no mAb). Inhibition of lysis was tested after preincubation of CTL clones with anti-CD3 mAb (OKT3; αCD3) or of the tumor target with anti-HLA-A2 mAb (CR11.351; αHLA-A2). ND, not done.

Table 2. Specificity of antigen recognition by HLA-A2-restricted anti-melanoma CTL clones

	TNF release by CTL clones, pg/ml								
	Patient 8959								Patient 9742
Stimulating cell	1A9/4	1A77	2A9	2A13	2A27	2A37	2A22	2A121	A81
Melanoma									
8959	67	32	56	72	11	68	18	39	33
9742	37	31	ND	>100	13	41	6	5	43
LB39	ND	70	ND	>100	23	ND	ND	ND	44
SK29.1.22	0	0	0	0	1	1	3	2	0
MZ2A2.1	ND	3	ND	0	ND	ND	ND	1	2
MZ2A2.1 Melan-A/MART-1	ND	32	ND	72	ND	ND	ND	0	44
COS-7 expressing									
A2.1	4	0	1	1	2	7	3	3	1
Melan-A/MART-1	2	0	0	0	ND	ND	0	ND	0
A2.1 Melan-A/MART-1	3	80	67	>100	22	5	2	5	63
A2.1 tyrosinase	4	ND	ND	3	ND	73	3	5	2
A2.1 MAGE-1	4	ND	ND	2	ND	5	3	5	1
A2.1 MAGE-2	3	ND	ND	2	ND	6	3	6	2
A2.1 MAGE-3	4	ND	ND	2	ND	6	3	5	0

TNF secretion by CTL clones coincubated with melanomas or transiently transfected COS-7 cells. 8959, 9742, and LB39, HLA-A2$^+$ melanomas; SK29.1.22, HLA-A2$^-$ melanoma variant; MZ2A2.1, stable MZ2-Mel (HLA-A1$^+$) transfectant expressing HLA-A2.1 but not Melan-A/MART-1; MZ2A2.1 Melan-A/MART-1, expresses HLA-A2 and Melan-A/MART-1. COS-7 cells were transfected with expression vector constructs containing full-length tyrosinase, Melan-A/MART-1, and MAGE-1, -2, and -3 cDNAs along with HLA-A2.1 cDNA. ND, not done.

2A121 produced TNF only in response to the autologous tumor, and the unique pattern of recognition was confirmed by their inability to lyse a panel of normal or neoplastic target cells in a ^{51}Cr-release assay, including HLA-A2$^+$ and HLA-A2$^-$ melanoma lines (data not shown). Sequence analysis indicated that they share the same V and J regions in their α and β chains (TCRAV3S1J55C1 and TCRBV8J2S3C1) and differ only at the V–J and V–D–J junctions (where D is the diversity region) (data not shown).

TCR Repertoire of Melan-A/MART-1-Specific CTL Clones Obtained from Different HLA-A2$^+$ Melanoma Patients. By grouping CTL clones on the basis of antigen recognition (Table 3), it was apparent that all Melan-A/MART-1-specific clones from patients 9742 and 8959 expressed TCRAV2 and four out of five (2A13, 2A27, 2A9, and A81) displayed the preferential TCRAV2/TCRBV14 combination. The TCR composition of additional Melan-A/MART-1 HLA-A2-restricted CTL clones, obtained from either tumor infiltrating lymphocytes or PBLs in different laboratories (14, 15, 17), is also shown in Table 3. CTL clone 1/95 from patient LB39 indeed expressed both VB14 and VA2 although a second α-chain V-region transcript (VA8) was also present. All CTL clones from patients AV and 501 expressed VB7 in their β chain joined to VA2 (10/196), VA1 (2/9), VA21 (A42), and either VA21 or VA10 (11/33). These data thus indicate that VA2 constitutes the majority of the α-chain V-region repertoire, being expressed in 7 of 10 CTL clones from four patients. Two predominant β-chain V regions were found, VB14 expressed in 5 out of 10 clones (three patients) and VB7 expressed in 4 of 4 clones from patients AV and 501. VB7 usage appears to be a consistent feature in patient AV since CTL clones 10/196 and 11/33 were obtained 8 years later than CTL clone 2/9 (17).

Table 3. Correlation between TCR usage and Melan-A/MART-1 recognition in HLA-A2-restricted CTL clones recognizing melanocyte differentiation antigens

Patient	CTL clone	Antigen recognition	TCRAV usage	TCRBV usage
8959	2A37	Tyrosinase	21	5
	1A9/4	Not identified	ND	6
	1A77	Melan-A/MART-1	2	4
	2A13	Melan-A/MART-1	2	14
	2A9	Melan-A/MART-1	2	14
	2A27	Melan-A/MART-1	2	14
9742	A81	Melan-A/MART-1	2	14
LB39	1/95	Melan-A/MART-1	2 8	14
AV	10/196	Melan-A/MART-1	2	7
	2/9	Melan-A/MART-1	1	7
	11/33	Melan-A/MART-1	21 10	7
501	A42	Melan-A/MART-1	21	7

ND, not done.

V–D and V–D–J Sequences of Melan-A/MART-1-Specific CTL Clones. Sequence analysis (Fig. 1) indicated that all clones except 2A13 and 2A9 arose from separate T cells. All VA2 clones expressed the same V-region family member VA2S1 (21). VB7 usage (26), instead, was more heterogeneous even in CTL clones 10/196, 2/9, and 11/88 derived from the same patient. Both TCR α chains found in CTL clones 1/95 (patient LB39) and 11/33 (patient AV) were productively rearranged as occurs in up to one-third of the mature T cells (34). In CTL clone 1/95, the α-chain-expressing VA2S1 is likely involved in tumor recognition being almost identical in its composition to the unique α chain used by clone 2A13/2A9 (patient 8959) except for a conservative amino acid substitution at the V–J junction (Fig. 1). The similarities between clones 1/95 and 2A13/2A9 are strengthened by usage of VB14 in their β chain and of an allelic variant of TCRBJ2S1 (30). With the exception of two clones (2A13/2A9 and 1/95), each of the others expressed a different TCRAJ or TCRBJ region and comparison of complementarity-determining regions 3 (CDR3s), defined according to Chothia *et al.* (33), did not indicate conservation in either length or amino acid composition. The first position of the CDR3 was, however, Asn in three out of six VA2S1-expressing clones. Similarly, in three out of four VB14 clones, a Gly was found at position 97 of their CDR3, whereas in VB7 clones, two negatively charged amino acids were conserved, a Gln at position 96 (present in all clones) and a Glu at position 97 (present in three out of four clones).

DISCUSSION

These results indicate that HLA-A2-restricted CTL clones derived from different patients and specific for the differen-

A

Patient	Clone	TCRA	V	CDR3	J	C
9742	A81	V2S1J9C1	TGTGCCGTG	AATACTGGAGGCTTCAAAACT	ATCTTTGGAGCAGGAACAAGACTATTTGTTAAAGCA	AATATCCAG
			C A V	N T G G F K T	I F G A G T R L F V K A	N I Q
8959	2A27	V2S1J45C1	TGTGCCTCA	GGAGGAGGTGCTGACGGACTC	ACCTTTGGCAAAGGGACTCATCTAATCATCCAGCCC	TATATCCAG
			C A S	G G G A D G L	T F G K G T H L I I Q P	Y I Q
8959	2A13	V2S1J35C1	TGTGCCGTG	AACATAGGCTTTGGGAATGTGCTG	CATTGCGGGTCCGGCACTCAAGTGATTGTTTTACCA	CATATCCAG
			C A V	N I G F G N V L	H C G S G T Q V I V L P	H I Q
8959	1A77	V2S1J39C1	TGTGCCGTG	AACCCAGGCAACATGCTC	ACCTTTGGAGGGGGAACAAGGTTAATGGTCAAACCC	CATATCCAG
			C A V	N P G N M L	T F G G G T R L M V K P	H I Q
LB39	1/95	V2S1J35C1	TGTGCCGTT	ACGATAGGCTTTGGGAATGTGCTG	CATTGCGGGTCCGGCACTCAAGTGATTGTTTTACCA	CATATCCAG
			C A V	T I G F G N V L	H C G S G T Q V I V L P	H I Q
		V8S1J48C1	TGTGCGGAC	TGGGGCCAATTTGGAAATGAGAAATTA	ACCTTTGGGACTGGAACAAGACTCACCATCATACCC	AATATCCAG
			C A D	W G Q F G N E K L	T F G T G T R L T I I P	N I Q
AV	10/196	V2S1J11C1	TGTGCCGTG	AAAGACAGCACCCTC	ACCTTTGGGAAGGGGACTATGCTTCTAGTCTCTCCA	GATATCCAG
			C A V	K D S T L	T F G K G T M L L V S P	D I Q
AV	2/9	V1S2J45C1	TGTGCTGTG	AGCCGAGGAGGAGGTGCTGATGGACTC	ACCTTTGGCAAAGGGACTCATCTAATCATCCAGCCC	TATATCCAG
			C A V	S R G G G A D G L	T F G K G T H L I I Q P	Y I Q
AV	11/33	V10J47C1	TGTGCAGGA	GCTCGGGAATATGGAAACAAACTG	GTCTTTGGCGCAGGAACCATTCTGAGAGTCAAGTCC	TATATCCAG
			C A G	A R E Y G N K L	V F G A G T I L R V K S	Y I Q
		V21J4C1	TGTGCAGCA	AGCCCGCCGGAATCTGGTGGCTACAATAAGCTG	ATTTTTGGAGCAGGGACCAGGCTGGCTGTACACCCA	TATATCCAG
			C A A	S P P E S G G Y N K L	I F G A G T R L A V H P	Y I Q
501	A42	V21J42C1	TGTGCCGCA	TATTATGGAGGAAGCCAAGGAAATCTC	ATCTTTGGAAAAGGCACTAAACTCTCTGTTAAACCA	AATATCCAG
			C A A	Y Y G G S Q G N L	I F G K G T K L S V K P	N I Q

B

Patient	Clone	TCRB	V	CDR3	J	C
9742	A81	V14D1J1S2C1	TGTGCCAGC	AGCACGGGACAGGGGTGGGGCTCC	TTCGGTTCGGGGACCAGGTTAACCGTTGTA	GAGGACCTG
			C A S	S T G Q G W G S	F G S G T R L T V V	E D L
8959	2A27	V14D1J2S2C2	TGTGCCAGC	AGTTTAGGGGTAGCGACCGGGGAGCTGTTT	TTTGGAGAAGGCTCTAGGCTGACCGTACTG	GAGGACCTG
			C A S	S L G V A T G E L F	F G E G S R L T V L	E D L
8959	2A13	V14D2J2S1C2	TGTGCCAGC	AGTCGGACTGTCGGGGGGCCCAATGAGCAGTTC	TTCGGGCCAGGGACACGGCTCACCGTGCTA	GAGGACCTG
			C A S	S R T V G G P N E Q F	F G P G T R L T V L	E D L
8959	1A77	V4J2S2C2	TGCAGCACT	GATGGGCAGACCGGCACCGGGGAGCTGTTT	TTTGGAGAAGGCTCTAGGCTGACCGTACTG	GAGGACCTG
			C S T	D G Q T G T G E L F	F G E G S R L T V L	E D L
LB39	1/95	V14D2J2S1C2	TGTGCCAGC	AGTCTTGGCAATGAGCAGTTC	TTCGGGCCAGGGACACGGCTCACCGTGCTA	GAGGACCTG
			C A S	S L G N E Q F	F G P G T R L T V L	E D L
AV	10/196	V7S1D1J1S5C1	TGCGCCAGC	AGCCAAGAAACCGACATCGTCTTCAATCAGCCCCAGCAT	TTTGGTGATGGGACTCGACTCTCCATCCTA	GAGGACCTG
			C A S	S Q E T D I V F N Q P Q H	F G D G T R L S I L	E D L
AV	2/9	V7S2D1J2S3C2	TGCGCCAGC	AGCCAAGGACAGCTCACAGATACGCAGTAT	TTTGGCCCAGGCACCCGGCTGACAGTGCTC	GAGGACCTG
			C A S	S Q G Q L T D T Q Y	F G P G T R L T V L	E D L
AV	11/33	V7S3J1S3C1	TGTGCCAGC	AGCCAAGAAGAGGGAGGAGGGTCTTGGGGAAACACCATATAT	TTTGGAGAGGGAAGTTGGCTCACTGTTGTA	GAGGACCTG
			C A .S	S Q E E G G G S W G N T I Y	F G E G S W L T V V	E D L
501	A42	V7S2D2J2S7C2	TGTGCCAGC	AGCCAAGAGGGACTAGCGGGAGCGTCGCAGTAC	TTCGGGCCGGGCACCAGGCTCACGGTCACA	GAGGACCTG
			C A S	S Q E G L A G A S Q Y	F G P G T R L T V T	E D L

FIG. 1. TCR α (*A*) and β (*B*) cDNA junctional nucleotide and amino acid sequences of Melan-A/MART-1-specific and HLA-A2-restricted CTL clones. The sequence of CTL clone 2A9, identical to that of 2A13, is not shown. For each clone, only the last three V-region gene residues are shown, followed by the presumed immunoglobulin-like loops (CDR3) defined according to Chothia *et al.* (33) and by the first 3 residues of the C region. TCRAV sequences are described in the following references: 21, AF110/TCRAV2S1; 22, HAP41/TCRAV8S1, HAP58/ TCRAV10; 23, PY14.2/TCRAV1S1; 24, L17Ti/TCRAV21. TCRBV chain sequences are described in the following references: 25, DT110/TCRBV4; 26, HT459/TCRBV7S1, HT267a/b/TCRBV7S2, HT267.2/TCRBV7S3; 27, ph21/TCRBV14. TCRAJ, TCRBD, J, and C elements are assigned according to germ-line sequences (28–30). TCRAV sequences are identical to the published sequences except for the following sequences: VA2S1, expressed in 2A13, 1/95, A81, and 10/196, differs by a single nucleotide substitution (Ser → Phe) at position 48 [numbering according to Chothia *et al.* (33)]; VA10, which differs at three positions, two of which are silent and the third results in a His → Leu replacement at position 87; VA21, expressed in clone A42 and differs for a silent substitution at position 91; JA45, which differs at a silent nucleotide substitution (double underlined). VB4 differs from the reported sequence at position 94. The TCRBJ2S1 segment containing the amino acid Thr double underlined (2A13 and 1/95) has been reported (30).

tiation antigen Melan-A/MART-1 display limited V-region usage in their TCRs. Identification of the antigens recognized by CTL clones derived from PBLs of patient 8959 was possible through the use of transient expression of HLA genes and cDNAs encoding melanoma antigens in COS-7 cells. Four out of eight CTL clones recognized Melan-A/MART-1, and one out of eight recognized tyrosinase. Unresponsiveness of three CTL clones on all COS-7 transfectants indicated the existence of at least two additional antigens, one of which is from the melanocyte lineage and the other is a melanoma-restricted antigen. The diversity of the antigens recognized on melanoma 8959 is not surprising given the large number of T-cell epitopes that can be expressed on autologous melanoma even considering only the HLA-A2-restricted response (35).

Although the TCR combinations expressed in CTL clones were diverse, the conservation of a few V-region families became apparent when they were grouped by antigen specificity. This can explain why other studies, performed without knowing the relevant antigen and the HLA molecule involved, failed to observe restricted TCRV gene usage in the T-cell response against melanoma (7, 9). The two clones directed against a putative melanoma antigen shared TCRAV3/TCRBV8, whereas all three anti-Melan-A/MART-1 CTL clones used TCRAV2 joined to TCRVB14 or TCRVB4. The analysis of other anti-Melan-A/MART-1 HLA-A2-restricted T-cell clones from four additional patients revealed that the majority of clones (six out of nine) expressed TCRAV2. Only three TCRBV families were used in all nine clones. It is not known whether the different Melan-A/MART-1-specific CTL clones recognize the same peptide from this antigen. Recently, recognition of four partially overlapping peptides of either 9 or 10 residues has been described for CTL clone A42 (36). These synthetic peptides correspond to a hydrophobic segment in the putative transmembrane region of the protein containing many possible HLA-A2-binding motifs (14, 15). It is, thus, possible that the restricted TCR V-region repertoire and the limited homology in the CDR3 regions of anti-Melan-A/MART-1 CTL clones may depend on recognition of distinct but perhaps overlapping peptides. The concept of a possible association of a few different TCR V regions with recognition of a well-defined human tumor antigen seen in the context of the same HLA is supported also by results in ovarian cancer (37).

These findings may lead to practical applications. Selection of $CD8^+$ T-cell populations, on the basis of TCRV gene expression, may be attempted to enrich for tumor-reactive CTLs in different $HLA\text{-}A2^+$ patients. In addition, the knowledge of the TCR structure of CTLs directed to a common melanoma antigen may also provide molecular markers to trace the evolution of immune response to the tumor *in vivo*.

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