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ORIGINAL ARTICLE

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Melanomas with concordant loss of multiple melanocytic differentiation proteins: immune escape that may be overcome by targeting unique or undefined antigens

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Abstract Melanoma-reactive HLA-A*0201-restricted cytotoxic T lymphocyte (CTL) lines generated in vitro lyse autologous and HLA-matched allogeneic melanoma cells and recognize multiple shared peptide antigens from tyrosinase, MART-1, and Pmel17/gp100. However, a subset of melanomas fail to be lysed by these T cells. In the present report, four different HLA-A*0201$^+$ melanoma cell lines not lysed by melanoma-reactive allogeneic CTL have been evaluated in detail. All four are deficient in expression of the melanocytic differentiation proteins (MDP) tyrosinase, Pmel17/gp100, gp75/trp-1, and MART-1/Melan-A. This concordant loss of multiple MDP explains their resistance to lysis by melanoma-reactive allogeneic CTL and confirms that a subset of melanomas may be resistant to tumor vaccines directed against multiple MDP-derived epitopes. All four melanoma lines expressed normal levels of HLA-A*0201, and all were susceptible to lysis by xenoreactive-peptide-dependent HLA-A*0201-specific CTL clones, indicating that none had identifiable defects in antigen-processing pathways. Despite the lack of shared MDP-derived antigens, one of these MDP-negative melanomas, DM331, stimulated an effective autologous CTL response in vitro, which was restricted to autologous tumor reactivity. MHC-associated peptides isolated by immunoaffinity chromatography from HLA-A1 and HLA-A2 molecules of DM331 tumor cells included at least three peptide epitopes recognized by DM331 CTL and restricted by HLA-A1 or by HLA-A*0201. Recognition of these CTL epitopes cannot be explained by defined, shared melanoma antigens; instead, unique or undefined antigens must be responsible for the autologous-cell-specific anti-melanoma response. These findings suggest that immunotherapy directed against shared melanoma antigens should be supplemented with immunotherapy directed against unique antigens or other undefined antigens, especially in patients whose tumors do not express MDP.

Key words Melanoma · Antigens · Cytotoxic T lymphocytes · Human · Immunotherapy

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Introduction

Melanoma-reactive cytotoxic T lymphocyte (CTL) lines can be generated in vitro by stimulation of human lymphocytes with autologous tumor in the presence of interleukin-2 (IL-2). These CTL recognize autologous melanoma cells, as well as most class-I-MHC-matched allogeneic melanomas, indicating that they recognize class-I-MHC-restricted melanoma antigens that are expressed on multiple tumors [12]. Many of the shared antigens restricted by HLA-A*0201 are derived from the melanocytic differentiation proteins (MDP) tyrosinase, Pmel17/gp100, and MART-1/MelanA. Tumor infiltrating lymphocytes with reactivity against these antigens can be expanded in vitro, and are able to mediate tumor regressions when administered to patients [20].

A growing understanding of the nature of melanoma antigens has led to initiation of clinical trials investigating immunotherapy directed against shared antigens derived from MDP [10, 12, 19, 20, 30]. However, approximately 30% of metastatic melanomas fail to express the MDP gp100, tyrosinase, and MART-1 [13] and, even in patients whose tumors express these antigenic proteins, there is heterogeneity, so that some tumor cells may fail to express them. Thus, a substantial proportion of melanomas in vivo may be resistant to immunotherapy directed at shared antigens derived from MDP. A rational approach to inducing tumor-specific immunity in patients whose melanomas have lost expression of the MDP is to use polyvalent vaccines incorporating other shared antigens such as the cancer/testis antigens [9], and/or antigens unique to the autologous tumor, through the use of polyvalent vaccine preparations.

For active specific immunotherapy with T cell antigens to be effective against antigen-loss melanomas, however, MHC expression and antigen-processing pathways must be intact in the tumor cells. In a few described cases, down-regulation of class I MHC expression and defective antigen processing have been described in human tumor cells [24]. Presumably these findings represent mechanisms by which tumor cells escape immune destruction when antigenic proteins are still expressed. However, it is unclear how often these immune-escape mechanisms may exist in conjunction with loss of antigen expression. In the present study, we have evaluated in detail four melanoma cell lines that are not lysed by polyclonal HLA-A*0201-restricted melanoma-reactive CTL. The initial intent was to determine what combination of defects permitted them to avoid CTL-mediated destruction and to assess whether there may be mechanisms by which immune recognition of these variant melanomas may be maintained. In each case, tyrosinase, gp100, MART-1, and gp75 are all undetectable, but MHC expression and antigen-processing machinery are intact. A CTL line generated by autologous tumor stimulation, in one of these cases, recognized autologous tumor only. We provide evidence that the reactivity of these CTL is directed against several unique or undefined antigens restricted by HLA-A1 and by HLA-A2. An implication is that design of optimal polyvalent immunotherapeutic strategies may require inclusion of unique antigens or other undefined antigens.

Materials and methods

Cell lines and HLA typing

All tumor cell lines were of human origin. Melanoma cell lines A375, HMCB (Bowes melanoma), C32, HT144, Malme-3M, SkMel-5, and SkMel-24 were obtained from the American Type Culture Collection (Bethesda, Md.). Na8Mel was obtained from Vincent Brichard and Thierry Boon. Melanoma lines DM6, DM13, DM14, DM93, DM281, and DM331 were established from patients with melanoma resected at Duke University. VMM1, VMM5, VMM12, VMM15, VMM17, and VMM18 are melanoma cell lines established from metastatic melanoma resected from patients at the University of Virginia. K562 is a natural-killer-cell(NK)-sensitive human erythroleukemia line. JY is an HLA-A*0201$^+$ Epstein-Barr-virus-transformed B lymphoblastoid line [15]. T2 is a mutated human HLA-A*0201$^+$ lymphoid cell line that has defects in the class-I-MHC-antigen processing pathway [27].

HLA types of the specimens used in this study (Table 1) were obtained by microcytotoxicity assay, either of the tumor cells or of autologous lymphocytes. Levels of expression of serological HLA-A2 on the tumor cells was determined by staining the tumor cells with BB7.2, a monoclonal antibody with specificity for the A2 molecule (American Type Culture Collection, ATCC, Rockville, Md.). Expression of the A2 subtype HLA-A*0201 was determined by staining with the HLA-A*0201/A*0202-specific monoclonal antibody CR11.351 [26] and by evaluating susceptibility to lysis by HLA-A*0201-specific murine CTL clones AT1-5 and AH III 12-2 [4, 18]. These CTL respectively recognize transporter-associated with antigen processing (TAP)-dependent and TAP-independent peptide epitopes associated with HLA-A*0201, where those peptide epitopes are expressed on most human cells [18].

Two HLA-A*0201-negative melanoma cell lines, HT144 and DM14, were transfected with the gene for HLA-A*0201 as previously described [31]. The transfectants HT144.A2-03 and DM14.A2-17 (from parental tumors HT144 and DM14, respectively) express HLA-A*0201 at levels comparable to those of melanoma cells that are naturally HLA-A*0201$^+$ (data not shown).

Throughout this report, the terms HLA-A2 and HLA-A*0201 are used with specific meaning and are not meant to be interchangeable. Cells described as HLA-A2$^+$ are those for which there is serological expression of HLA-A2, but no direct evidence that the allele expressed is HLA-A*0201. Where a cell line is described as HLA-A*0201$^+$, there has been direct evaluation of expression of that allele, based on one or more of the methods described above.

Melanoma-reactive CTL lines

The generation and characterization of VMM5 CTL and DM252-6 CTL have been previously reported [10, 11, 31]. Both of these CTL lines were generated in vitro from melanoma patients, by repeated stimulation with HLA-A*0201$^+$ melanoma cells. They recognize shared antigens on HLA-A*0201$^+$ melanomas, including the Pmel-17/gp100-derived peptides YLEPGPVTA [10] and KTWGQYWQV [3, 20], Mart-1/Melan-A-derived peptide AAGIGILTV [19], and additional peptides not yet identified [29, 31].

The VMM15 CTL line was generated from lymph node lymphocytes from melanoma patient VMM15, restimulated in vitro repeatedly with autologous tumor only. These CTL have been characterized as recognizing shared antigens on HLA-A1$^+$ melanomas, and with specific reactivity for a tyrosinase peptide, restricted by HLA-A1 [21].

Table 1 HLA typing of human cell lines used in this study. HLA typing on VMM1, VMM5, VMM6, VMM12, VMM13, VMM15, VMM17, and VMM18 was performed by microcytotoxicity assay on autologous lymphocytes (Gentrak). The expression of HLA-A*0201 on tumor cells was confirmed by specific antibody staining of the tumor cells with monoclonal antibodies BB7.2 and CR11.351. HLA types of the lines from the ATCC are reported by the ATCC. Where no other HLA types were known, expression of A*0201 was determined by staining with BB7.2 and/or CR11.351. Where HLA-A*0201 is cited specifically, the subtype has been defined by susceptibility to HLA-A*0201-specific murine cytotoxic T lymphocytes, as described in this manuscript. HLA types of the other cell lines have been reported [17]. –None reported or not evaluated, *ND* none detected, either by microcytotoxicity assay or by staining with monoclonal antibodies

Cell line	Cell type	Class I MHC			Class I MHC	
		HLA-A	HLA-B	HLA-C	HLA-DR	HLA-DQ
A375	Melanoma	*0201	–	–	4, 7[b]	*0302(8), *0303(9)[b]
HMCB(Bowes)	Melanoma	2	–	–	–	–
C32	Melanoma	2	–	–	–	–
DM6	Melanoma	*0201	12, 13	1, 2	6, 7, 10[a]	–
DM13	Melanoma	*0201, 31	13, 18	ND	ND	ND
DM14	Melanoma	11, 68	5, 8	2, 4	–	–
DM14.A2-17	Melanoma	*0201, 11, 68	5, 8	2, 4	1, 1[b]	*0501(5), *0501(5)[b]
DM93	Melanoma	*0201, 33	8, 49	ND	3/6, 3/6[b,c]	*0603(6), *0604(6)[b,c]
DM281	Melanoma	2	–	–	–	–
DM331	Melanoma	1, 2	15, 62	–	1, 4[b]	*0302(8), *0501(5)[b]
HT144	Melanoma	1, 24	13	3	–	–
HT144.A2-03	Melanoma	1, *0201, 24	13	3	–	–
JY	Lymphoblast	*0201	7	–	4, 6	–
K562	Erythroleukemia	–	–	–	–	–
Malme-3M	Melanoma	2	13, 40	–	–	–
Na8Mel	Melanoma	2	–	–	–	–
SkMel5	Melanoma	2, 11	7	–	–	–
SkMel24	Melanoma	1, *0201	12, 14	–	1, 3/6[b]	*0502(5), *0604(6)[b]
T2	Lymphoid	*0201	–	–	–	–
VMM1	Melanoma	3, 26	51	ND	1, 2[b]	–
VMM5	Melanoma	*0201	39	ND	7, 11	2, 7
VMM6	Melanoma	*0201, 33	14, 44	–	–	–
VMM12	Melanoma	1, 3	7, 14	ND	–	–
VMM13	Melanoma	1, 2	53	–	–	–
VMM15	Melanoma	1, 25	8, 18	ND	–	–
VMM17	Melanoma	*0201, 33	45, 55/66	ND	–	–
VMM18	Melanoma	3, 31/33	60	3	2, 9[b]	–

[a] Three DR antigens are listed because cross-reactivity prevented ascertaining with certainty which two were correct
[b] HLA typing of class II molecules performed by Dr. Jeff E. Lee at MD Anderson by oligotyping methods; DQB1 alleles are listed
[c] Previously reported as DR2,4,6; DQ3

Murine CTL specific for the naturally processed HLA-A*0201-associated tyrosinase peptide YMDGTMSQV [10, 28] were generated by immunization of HLA-A2 (AAD) transgenic mice with a vaccinia construct encoding that epitope via a minigene directionally ligated into the poly-linker site of the plasmid pSC11.3. After immunization, splenocytes were harvested, then restimulated weekly in vitro with the purified peptide at 1 μg/ml in the presence of 10 units/ml IL-2. These CTL specifically recognize the YMDGTMSQV peptide, whether pulsed onto HLA-A*0201$^+$ cells or expressed endogenously [22]. HLA-2 (AAD) transgenic mice express HLA-A*0201 molecules expect that the α_3 domain has been replaced by the α_3 domain of the murine H-2k^d molecule.

Cytotoxicity assays

^{51}Cr-labelled target cells were plated at 2×10^3 cells/well in triplicate in 96-well V-bottom plates (Costar, Cambridge, Mass.) with varying numbers of effector cells in a final volume of 200 μl. Wells containing either culture medium and target cells only or 1 M HCl and target cells served as background and total release controls respectively. The plates were centrifuged at 100 *g* for 3 min and incubated at 37 °C in 5% CO_2 for 4 h. The plates were again centrifuged, and 150 μl medium from each well was removed for counting in a gamma counter. Percentage specific ^{51}Cr release was calculated as [^{51}Cr (experimental) $-^{51}$Cr (background)] × 100/[^{51}Cr (total release) $-^{51}$Cr (background)].

Reconstitution of CTL epitopes by addition of synthetic or naturally processed peptides

Synthetic peptides (1 μg/ml) were incubated for 2 h at 37 °C with 2×10^3 ^{51}Cr-labelled tumor cells in 100 μl assay medium (RPMI, 10% fetal calf serum, antibiotics)/well in 96-well plates. Effector cells were then added in 100 μl assay medium, and the remainder of the assay was performed as described above. Wells containing peptide and target cells, but no CTL, were used as controls to rule out toxicity of the peptides themselves.

Western analysis

The antibody specificities for these analyses included gp100/Pmel-17 (HMB45, BioGenex, San Ramon, Calif.), tyrosinase (T311, kindly provided by Elisabeth Stockert and Lloyd J. Old, Ludwig Institute for Cancer Research, Memorial Sloan-Kettering Cancer Center, New York, N.Y.), TAP1 (kindly provided by Robert Tampe, Max-Planck-Institute, Martinsried, Germany), and actin (Chemicon, Temecola, Calif.).

Between 8×10^6 and 1.3×10^7 cells were incubated for 1 h on ice in a tenfold excess (v/v) of lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 10 mM TRIS pH 7.5, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin). After centrifugation at 30 000 *g* for 30 min, the supernatant was diluted in sample buffer [0.1% bromophenol blue, 2% glycerol, 124 mM TRIS pH 6.8, and 4%

sodium dodecyl sulfate (SDS)]. (For T311 and anti-TAP1 only, 10% 2-mercaptoethanol was also added, and the samples were boiled for 5 min prior to loading.) The equivalent of 0.5×10^6 cells was loaded into each well of a 10% SDS/polyacrylamide gel. After electrophoresis, the protein was transferred to Protran nitrocellulose (Schleicher and Schüll, Keene, N.H.) using a semidry apparatus at 15 V for 1 h. The membrane was blocked, incubated with primary antibody overnight, then washed. After incubation with sheep anti-(mouse Ig) secondary antibody for 3 h, chemiluminescent substrate was added (ECL Western Blotting Analysis System Amersham Life Science, Buckinghamshire, England). The membrane was then exposed to film.

Immunohistochemistry

Immunohistochemical evaluation of tumor cells was also performed, to determine their expression of known melanocyte/melanoma lineage proteins. Tumor cells were plated onto glass slides, then fixed and stained by the immunoperoxidase method, using the Vectastain Elite ABC reagent kit (Vector Laboratories, Burlingame, Calif.). The specificities evaluated included gp100/Pmel-17 (HMB45), tyrosinase (T311), MART-1/Melan-A (A103, kindly provided by Elisabeth Stockert), S100 (BioGenex Laboratories, San Ramon, Calif.), epithelial membrane antigen (Dako Corporation, Carpinteria, Calif., clone E9), a cytokeratin cocktail of AE1/AE3 (Boehringer-Mannheim CBA304), CAM5.2 (Becton-Dickinson M0910), 902 (Enzo 8KDA2), 903 (Enzo 7EFB1), and MAK-6 + Triton (GJ1001A), and vimentin (Biogenex Laboratories, San Ramon, Calif., clone V9). The antibody used for TRP-1/gp75 (MAB1644, Chemicon, Temecula, Calif.) is described by the manufacturer as an antibody to T4 tyrosinase [6].

RNA isolation and Northern blot analysis

Messenger RNA was extracted from melanoma cells. A 10-μg sample of RNA was denatured, electrophoresed through a 1% agarose gel, and transferred to Optitran-supported nitrocellulose membrane (Schleicher and Schüll), which was hybridized with one of three ^{32}P-labelled probes. The tyrosinase probe encompasses nucleotides 24–1382 of the tyrosinase coding region. The Pmel17/gp100 probe encompasses the full length of the Pmel17/gp100 open reading frame and an additional 204 nucleotides of pSC11.3. The probe used as a positive control was a 1.1-kb fragment of chicken glutaral-dehyde dehydro-genase (GAD) (kindly provided by Dr. Tim Bender). Hybridization was performed overnight in 50% w/v formamide/5× standard saline citrate/5× Denhardt's solution (GSB, Cleveland, Ohio) at 42 °C with gentle shaking. The blots were washed at room temperature, then exposed to phosphoimager cartridges for 24–28 h and analyzed with ImageQuant software.

RNA extraction, cDNA synthesis, and the polymerase chain reaction (PCR) to evaluate protein expression in melanomas

RNA of melanoma cell lines was prepared using Trizol (Gibco BRL) according to the manufacturer's instructions. The RNA was subsequently analyzed for tumor-associated antigen (TAA) expression by reverse transcription and PCR amplification (RT-PCR) with TAA-specific oligonucleotide primers spanning introns in their genomic sequences, thus ensuring mRNA-specific amplification. cDNA synthesis was performed with 2 μg total RNA. PCR amplification was performed in a total volume of 100 μl containing 1/5 volume of the cDNA solution, 10 μl 10× PCR buffer (Perkin-Elmer), 8 μl each of 2.5 mM dNTP mix, 1 μl each of 20 μM solutions of the sense and antisense primers for the respective TAA and 2.5 units of Taq polymerase (Perkin-Elmer). The following sense and antisense primers were utilized:

MAGE-1, CHO14 (CGGCCGAAGGAACCTGACCCAG) and CHO12 (GCTGGAACCCTCACTGGGTTGCC) [7]

MAGE-3, (TGGAGGACCAGAGGCCCC) and (GGACGATTATCAGGAGGCCTGC) [16]

BAGE, VDB85 (TGGCTCGTCTCACTCTGG) and VDB86 (CCTCCTATTGCTCCTGTTG) [5]

GAGE, VDE24 (CCATCAGGACCATCTTCA) and VDE18 (AGACGCTACGTAGAGCCT) [32]

Melan-A/MART-1, (AGACGAAATGGATACAGAGC) and (CATGATTAGTACTGCTAGCG) [2]

Tyrosinase, (TTGGCAGATTGTCTGTAGCC) and (AGGCATTGTGCATGCTGCTT) [2]

gp100, (TGCATCTTCCCTGATGGTGGAC) and (GGAGTTGACATCTCTGCCAGTGTG) [1]

p15, M2a (CAACAACGACAAGCTCTCCAAGAG) and M2b (GGAACACTGCCGCAAACGTC) [25].

Amplification of β-actin cDNA was also performed to check the integrity of the RNA sample. The PCR products were resolved on an agarose gel and revealed by ethidium bromide staining.

Isolation of naturally processed HLA-A1- and HLA-A2-associated peptides

HLA-A1- and HLA-A2-associated peptides were acid-eluted, respectively, from HLA-A1 and HLA-A2 molecules affinity-purified from melanoma cells, by methods previously described [10]. Briefly, 2×10^{10} DM331 melanoma cells (HLA-A1, A2, B15, B62, Table 1) cultured in cell factories (Nunc, Naperville, Ill.) collected after trypsinization, then were washed three times in cold phosphate-buffered saline, pelleted, then snap-frozen. Cell pellets were detergent-solubilized in 1% CHAPS detergent, 174 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 10 μg/ml leupeptin, 16 μg/ml pepstatin A, 0.2% sodium azide and 0.03 μg/ml EDTA for 1 h at 4 °C. After centrifugation at 100 000 *g* for 1 h at 4 °C, the pellet of insoluble proteins was discarded, and the supernatant was filtered (0.2 μm) then sequentially passed over three protein-A–Sepharose columns. The first column was pre-coated with mAb B1.23.2 to deplete products of the HLA-B and C loci, the second column was pre-coated with mAb BB7.2 to collect the HLA-A2 molecules, and the third column was pre-coated with the pan-reactive anti-(class I) mAb W6/32 (ATCC) to bind the remaining MHC class I molecule, HLA-A1. HLA molecules and associated peptides were eluted from each column with 0.2 M acetic acid, pH 2.7, and the peptides were dissociated at pH 2.1 by bringing the solution to 10% acetic acid and boiling for 5 min. Finally, peptides were centrifuged through Ultrafree-CL 5000-kDa filters (Millipore, Bedford, Mass.) at 2500 *g* for 5 h. Filtrates containing purified peptides were concentrated by vacuum centrifugation and were stored at −80 °C.

HPLC fractionation of naturally processed peptides

Extracted HLA-A1- and HLA-A2-associated peptides from DM331 melanoma cells were fractionated on a Higgs (Mountainview, Calif.) reversed-phase C18 column (5-μm particles, 30 nm pore size, 2.1 mm inner diameter, 4 cm length). Solvent A was 0.1% trifluro acetic acid in water; solvent B was 0.085% trifluro acetic acid and 60% acetonitrile in water. The gradient consisted of 100% solvent A (0–5 min), 100% solvent A to 15% solvent B (5–10 min), 15% to 60% solvent B (10–60 min), and 60% to 100% solvent B (60–67 min) at a flow rate of 200 μl/min. Fractions were collected at 40-s intervals from 5 min to 67 min. These fractionated peptides were evaluated in CTL reconstitution assays as described above.

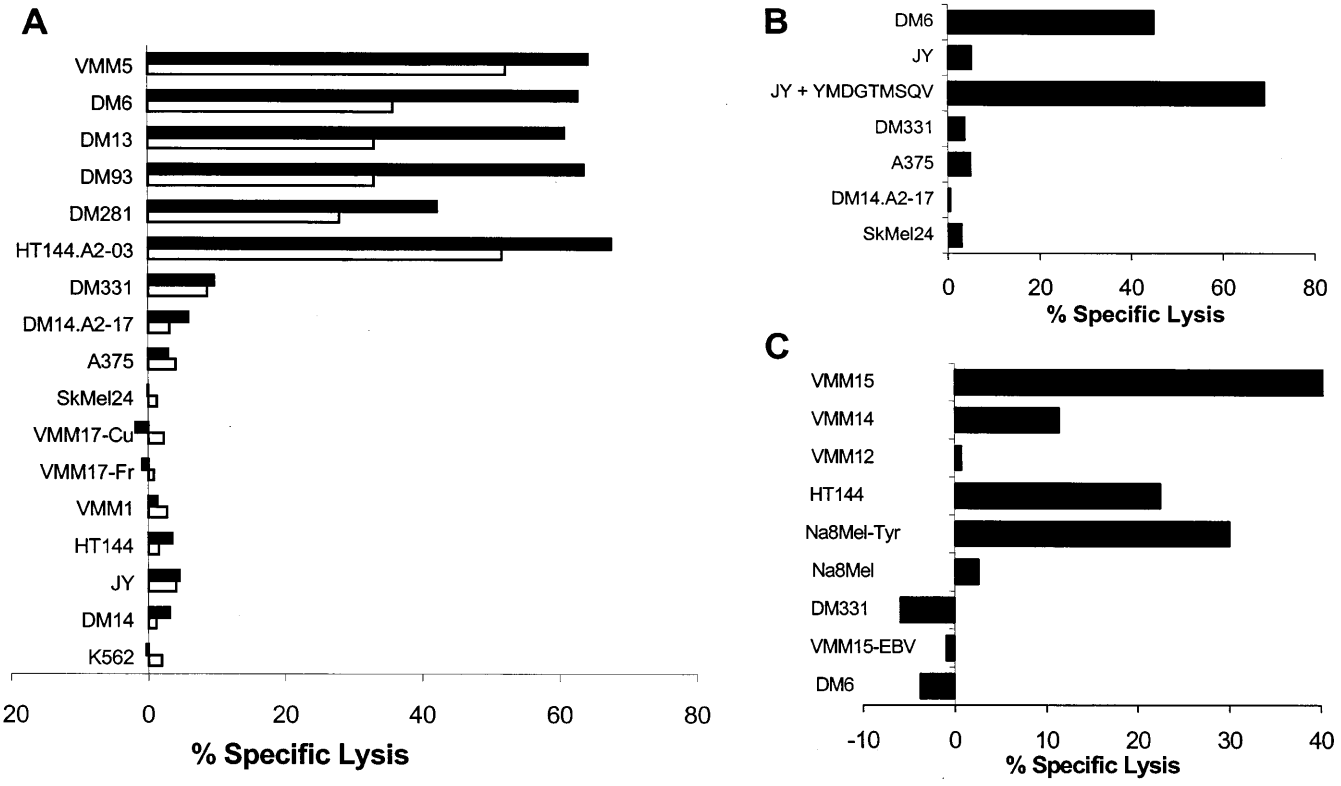
Results

Four melanomas fail to be recognized by allogeneic HLA-A*0201-restricted, melanoma-reactive CTL

Two HLA-A*0201-restricted CTL lines reactive against multiple shared melanoma antigens were evaluated for

their ability to lyse a panel of HLA-A*0201$^+$ melanomas. The HLA-A*0201-restricted VMM5 CTL line has been generated by stimulation of patient lymphocytes with autologous HLA-A*0201$^+$ tumor cells and restimulation with cells of HLA-A*0201-matched melanoma DM6, specifically to develop CTL reactivity against shared antigens [10]. It is a polyvalent CTL line reactive to HLA-A2-restricted CTL epitopes from gp100, MART-1/Melan-A, and other as yet undefined shared antigens [10, 29]. These CTL lysed six different A*0201$^+$ melanoma cell lines tested, including DM6, DM93, DM13, DM281, VMM5 (autologous), and HT144.A2-03, but failed to lyse the five A*0201$^+$ melanomas SkMel24, A375, DM14A2-17, DM331, and VMM17 (Fig. 1A). Both cultured and fresh tumor cells were available from VMM17; neither type of cell was lysed (Fig. 1A). An identical pattern of reactivity was observed when a different CTL line, DM252–6 CTL (Fig. 1A) was used. In other assays, five additional HLA-A2$^+$ melanomas VMM6, VMM13, Sk-Mel-5, C32, and Malme-3M were also lysed, and two additional A2$^+$ melanomas, Na8Mel, and HMCB (Bowes) failed to be recognized by VMM5 CTL (data not shown). Similarly, none of the four nonrecognized melanomas SkMel24, A375, DM14A2-17, and DM331 was lysed by an A2-transgenic murine CTL line specific for the tyrosinase peptide YMDGTMSQV [10, 28], presented in association with HLA-A*0201 (Fig. 1B). In addition, HLA-A1-restricted VMM15 CTL (reactive to a shared tyrosinase-derived peptide [21]) recognized several autologous and allogeneic HLA-A1$^+$ melanomas (VMM15, VMM14, and HT144) but failed to lyse the HLA-A1$^+$ melanomas VMM12, Na8Mel, and DM331 (Fig. 1B). VMM12 tumor cells do not stably express HLA-A1 (data not shown). Na8Mel is tyrosinase-negative, but the tyrosinase-transfected Na8Mel-Tyr is lysed by these CTL. DM331 is an HLA-A1$^+$, HLA-A*0201$^+$ melanoma not recognized by melanoma-reactive CTL restricted either by HLA-A1 or HLA-A*0201. Thus, the failure of several melanoma cell lines to be recognized by these CTL suggested either that the cell lines lack all of the relevant epitopes from MART-1/Melan-A, gp100, and tyrosinase, or that there is some other characteristic of these cells that globally affects T cell recognition, such as lack of MHC expression or a defect in antigen processing.

Fig. 1A–C HLA-A*0201-restricted and HLA-A1-restricted melanoma-reactive cytotoxic T lymphocyte (CTL) lines fail to recognize a subset of HLA-A*0201$^+$ melanomas. In 4-h ^{51}Cr-release assays, lysis of melanoma and non-melanoma targets by four different CTL lines was evaluated. **A** Melanoma-reactive VMM5 CTL (line E2.3, *empty bars*) and DM252-6 CTL (*solid bars*) were assayed (E:T = 10:1) on the indicated targets. Except for JY and K562, the target cells are all melanomas. VMM17-Cu is the cultured VMM17 melanoma cell line, and VMM17-Fr is the fresh cryopreserved VMM17 tumor cell suspension. The other melanomas are all human tumor lines. All target cells except VMM1, HT144, and DM14 were HLA-A2$^+$. **B** HLA-A2 transgenic murine CTL that recognize the tyrosinase peptide YMDGTMSQV in association with HLA-A*0201 were assayed (E:T = 30:1). JY cells are a negative control, and JY cells pulsed with the tyrosinase-derived peptide (YMDGTMSQV) were used as positive controls. DM6 is a tyrosinase-positive, HLA-A*0201$^+$ melanoma that also served as a positive control. **C** HLA-A1-restricted human-melanoma-reactive VMM15 CTL were assayed (E:T = 20:1) on several HLA-A1$^+$ and HLA-A1$^-$ target cells



The four nonrecognized melanomas SkMel24, A375, DM14A2-17, and DM331 were further characterized to determine the mechanism for their escape from CTL recognition. Available histological and clinical data on the original tumors confirm their identity as melanomas. DM331 was established in 1993 from melanoma metastatic to a single right axillary lymph node, 2 years after resection of the primary lesion from the right chest wall; grossly it was pale to gray in color; immunohistochemical stains for HMB45 and S100 were both positive, with approximately 10%–15% of the melanoma cells in the original nodal metastasis staining with HMB45 (data not shown). The DM14 cell line, from which DM14.A2-17 was derived by transfection with the gene for HLA-A*0201, was propagated in 1975 from a lymph node metastasis of melanoma from a known primary, where this and other metastases contained melanin in patches. Subsequent immunohistochemical staining of a metastatic deposit revealed cytoplasmic staining with HMB45 in approximately 5% of the cells (data not shown). The A375 and SkMel24 cell lines were obtained from the American Type Culture Collection (ATCC) and, although specific pathology reports and pertinent clinical data were not obtained, these lines have been well-characterized as melanomas. SkMel24 was derived from a lymph node metastasis of melanoma; it forms malignant melanoma in the nude mouse [17]. A375 forms tumors resembling amelanotic melanomas in mice [17]. Thus, all four nonrecognized melanoma cell lines were derived from metastatic melanomas.

Nonrecognized melanomas fail to express multiple MDP

Three of the four nonrecognized melanoma lines are nonpigmented and one is only weakly pigmented (data not shown), whereas the melanomas that were recognised, DM6 and DM93, are prominently pigmented, suggesting that pigmentation-related proteins were absent. Tyrosinase and Pmel17/gp100 protein expression was evaluated by Western analysis. Pmel17/gp100 bands were found in the positive control lanes (DM6 and DM93), but were absent from all four nonrecognized melanomas (Fig. 2A). Similarly, tyrosinase bands were found in the positive control lanes (DM6 and DM93), and in Na8Mel$^+$tyr, a tyrosinase-positive transfectant of the tyrosinase-negative melanoma Na8Mel, but were absent from all four nonrecognized melanomas as well as from Na8Mel [28], which also is not recognized by murine CTL specific for the tyrosinase antigen YMDGTMSQV (Fig. 2B). MART-1 protein expression was evaluated in all four variant melanomas by immunohistochemical analysis. Staining was found in the positive control cell line (DM6) but was absent from all four nonrecognized melanomas (Fig. 3). Similarly, all four were negative for staining with TRP-1/gp75 antibodies, while 50%–95% of DM6 and DM93 melanoma cells were positive, and immunohistochemical staining

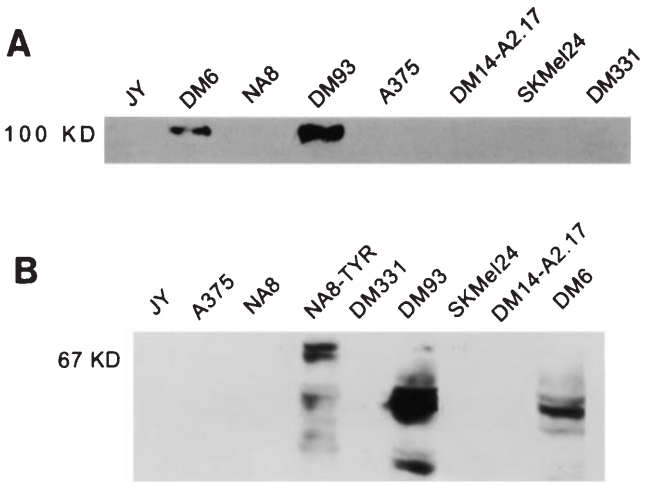


Fig. 2A, B Expression of Pmel17/gp100 and tyrosinase protein in nonrecognized melanomas. Western analysis for Pmel17/gp100 expression (**A**) and tyrosinase expression (**B**) was performed for four melanomas (A375, DM14.A2-17, DM331, and SkMel24). DM6 and DM93 were positive controls and lymphoblastoid cells JY were a negative control. Na8Mel$^+$Tyr, transfected with tyrosinase, also served as a positive control for tyrosinase expression. The Pmel17/gp100 gene product is represented by a 100-kDa band. The tyrosinase gene product is represented by several bands, presumably representing variations in the extent of post-translational modifications

for gp100 and tyrosinase was positive or strongly positive diffusely for DM6 and DM93 but negative for all four non-recognized melanomas (data not shown). Immunohistochemical analysis for the melanoma marker S100 was positive for all six melanomas tested, being strongly and diffusely positive for DM6 and SkMel24, moderately to strongly positive for DM93 and A375 and weakly positive for DM14.A2-17 and DM331 (data not shown). Thus, all of the nonrecognized melanomas were negative for all four of these MDP but positive for the S100 melanoma antigen.

Northern analysis was also performed to determine if mRNA for Pmel17/gp100 and tyrosinase was transcribed in any of the four nonrecognized melanomas. In both cases, bands hybridizing to the respective probes were evident in the positive control lanes (DM6 and DM93), but were absent from all four nonrecognized melanomas, as well as from Na8Mel (data not shown). As a control, ethidium bromide staining of the gel confirmed that equivalent amounts of RNA were loaded in each lane, and Northern analysis for GAD confirmed that equivalent amounts of RNA samples were transferred to the nitrocellulose blots (data not shown). Thus, neither Pmel-17/gp100 nor tyrosinase gene expression was detected in the four nonrecognized melanoma cell lines.

HLA-A*0201 expression by nonrecognized melanoma cells

Melanoma cells were stained with monoclonal antibody BB7.2 (anti-HLA-A2), CR11.351 [26] (anti A*0201 and

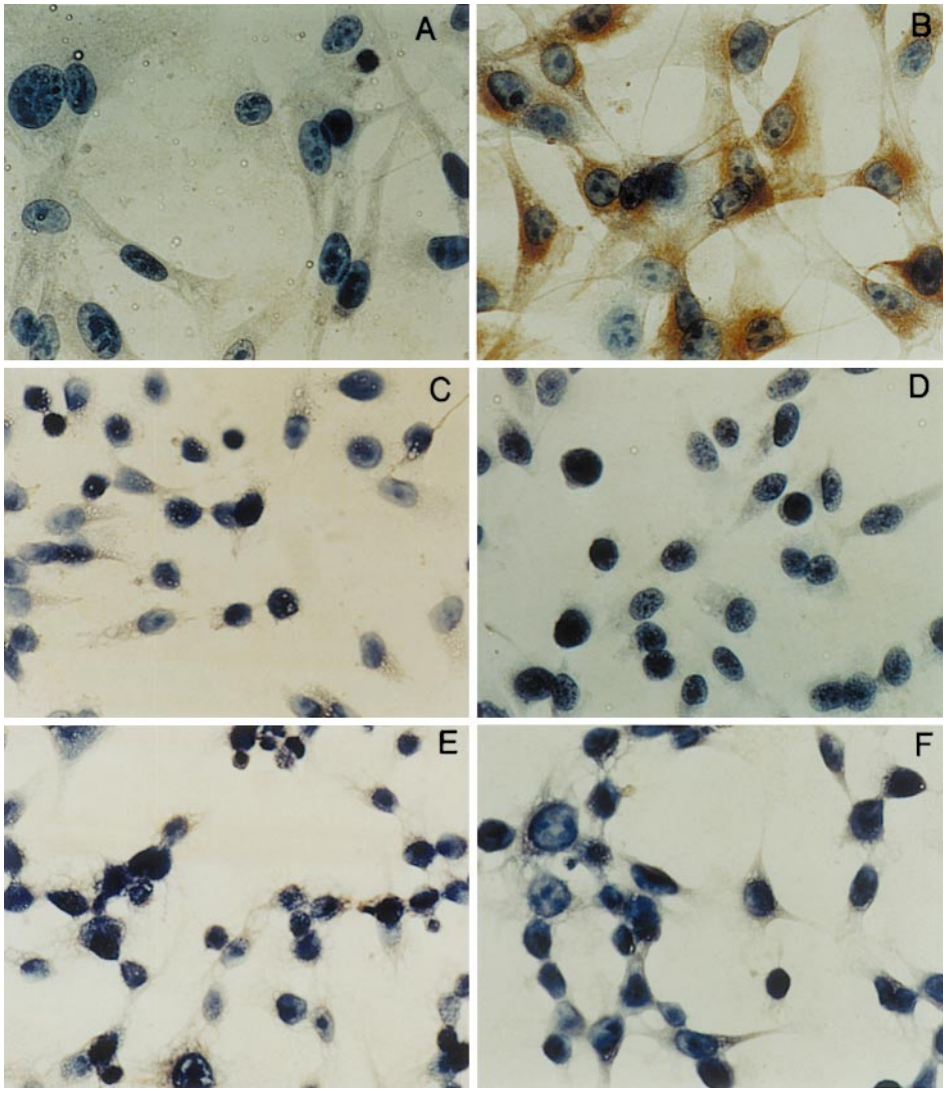


Fig. 3A–F Immunohistochemical analyses of MART-1/Melan-A protein expression. Cells were fixed on tissue-culture slides then stained with anti-MART-1/Melan-A antibody, A103. Negative controls were obtained by staining with the secondary antibody only. The chromagen was diaminobenzidine. Examples are shown for the positive control, DM6 (**A** negative control, **B** A103), and for the nonrecognized melanomas SkMel24 (**C** negative control, **D** A103), and DM14.A2-17 (**E** negative control, **F** A103).

A*0202), or W6/32, (all class I MHC) and evaluated by fluorescence-activated cell sorting. The lines evaluated included the HLA-A2^{neg} melanoma VMM18 as a negative control, the HLA-A2^{+} melanomas DM6 and DM93, which are recognized well by CTL, as positive controls, and the four nonrecognized melanomas SkMel24, A375, DM14A2-17, and DM331. All four nonrecognized melanoma lines expressed HLA-A*0201 molecules, and HLA class I molecules in general, at a level comparable to that of CTL-sensitive melanomas DM6 and DM93, with mean fluorescence values (arbitrary units) of approximately 50–300 for the nonrecognized melanomas, approximately 100 for the two recognized melanomas, and less than 10 for the negative control (data not shown). Thus there was no evidence, for any of these melanoma lines, for downregulation of class I MHC expression generally or for downregulation of HLA-A*0201 specifically.

Reconstitution of an epitope for HLA-A*0201-restricted melanoma-reactive CTL

Pre-incubation of SkMel24, A375, DM14.A2-17 with the Pmel17/gp100-derived peptide epitope YLEPGPVTA [10] reconstituted recognition by VMM5 CTL (Fig. 4). Comparable results have been observed also with DM331 (data not shown), and for all four melanomas pulsed with the MART-1 peptide AAGIGILTV (data not shown). Thus, none of these tumors is inherently resistant to lysis by human CTL, and all can present A*0201-associated peptides for recognition by A*0201-restricted CTL.

All four non-recognized melanomas have normal antigen processing

In order to determine whether the four non-recognized melanomas had defective antigen processing, in addition

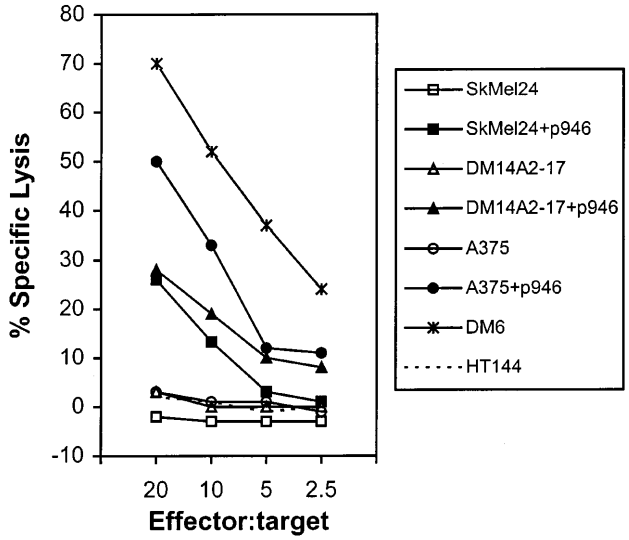


Fig. 4 Nonrecognized melanomas can present exogenously added peptide. The nonrecognized melanomas DM14A2.17 (△, ▲), SkMel24 (□, ■;), and A375 (○, ●), were preincubated either with 1 μg/ml Pmel17/gp100-derived peptide YLEPGPVTA (▲, ■, ●) or assay medium (△, □, ○) for 1 h prior to addition of effector cells (VMM5 CTL) at several effector:target ratios. The recognized melanoma DM6 (*) served as a positive control and the HLA-A2$^-$ melanoma HT144 served as a negative control (—)

Table 2 Susceptibility of melanomas to lysis by xenoreactive, A*0201-specific murine cytotoxic T lymphocyles (CTL). Values in bold type are considered positive

Target cell	Cell type/ expression of HLA-A2	Specific lysis (%) for the following CTL line and assay conditions	
		AT1-5 CTL E:T = 1:1	AHIII 12.2 CTL E:T = 10:1
DM93	Melanoma/A2$^+$	**30**	**87**
DM281	Melanoma/A2$^+$	**33**	**73**
DM14	Melanoma/A2$^-$	6	3
K562	Lymphoid/A2$^-$	0	0
A375	Melanoma/A2$^+$	**23**	**51**
DM14 A2.17	Melanoma/A2$^+$	**36**	**38**
DM331	Melanoma/A2$^+$	**62**	**74**
SkMel24	Melanoma/A2$^+$	**62**	**95**

to the loss of melanocytic tissue differentiation proteins, direct and indirect evaluations of the antigen-processing pathways were performed. The murine CTL clones AT1-5 and AHIII 12–2 recognize HLA-A*0201 in association with naturally processed peptides presented by TAP-dependent and -independent pathways, respectively [18]. Both of these CTL lysed each of the four nonrecognized cell lines, as well as other HLA-A*0201$^+$ melanomas (Table 2). These data confirm that both antigen-processing pathways are intact in all four melanoma cell lines tested.

These results further confirm that all four of the nonrecognized melanomas express the HLA-A*0201 subtype, because these CTL do not recognize the related HLA-A2 subtypes *0202, *0203, and *0205 [4]. As a more direct evaluation, Western analysis of TAP1 showed that its expression in these four nonrecognized melanomas was comparable to that of recognized cell lines (data not shown).

Autologous-cell-specific CTL can be generated from a patient with a nonrecognized melanoma, which lacks shared antigens

Autologous peripheral blood lymphocytes were available from one patient with a nonrecognized melanoma, DM331. This patient was originally diagnosed as having melanoma in 1991 and presented with metastatic melanoma in a single palpable axillary lymph node 2 years later, in August 1993. Since surgery, he has remained alive and well for 4 years, clinically free of disease since September 1997. DM331 CTL were generated by culturing these lymphocytes in IL-2-containing medium and repeatedly stimulating in vitro with autologous tumor cells. These DM331 CTL lysed autologous DM331 tumor cells but none of over a dozen allogeneic melanomas that share one or more MHC molecules with DM331, including four HLA-A1$^+$ melanomas and eight HLA-A*0201$^+$ melanomas (Fig. 5A–B). These DM331 CTL were restricted by class I MHC molecules, as shown by inhibition of autologous tumor lysis in the presence of the pan-class-I MHC monoclonal antibody w6/32 (Fig. 5C). Lysis of autologous tumor by DM331 CTL was not inhibited by antibodies to HLA-A1 (HA-1), HLA-A2 (BB7.2), or HLA-B/C (B1.23.2) individually, suggesting restriction by more than one MHC molecule (data not shown).

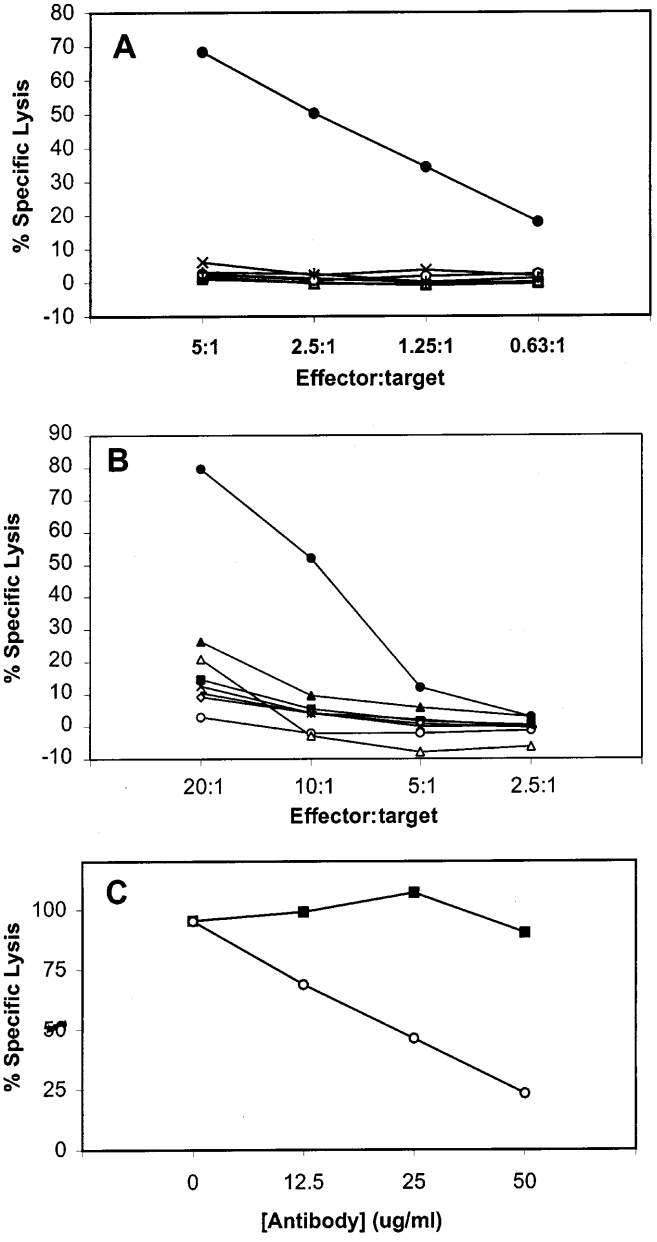
Purification of naturally processed peptides associated with HLA-A1 and HLA-A*0201 was accomplished by acid elution from the affinity-purified MHC molecules of DM331. Reconstitution of CTL epitopes for

►

Fig. 5A–C DM331 CTL lyse autologous tumor cells with class-I-MHC-restricted specificity. DM331 CTL were generated from peripheral blood lymphocytes by repeated stimulation in vitro with fresh, then cultured autologous DM331 melanoma cells. **A** After 64 days in culture, they were assayed for their ability to lyse autologous (HLA-A1$^+$, A2$^+$) DM331 melanoma cells (●), the NK target K562 (○), HLA-A*0201$^+$ lymphoblastoid cells JY (+), or any of four allogeneic melanomas: VMM12 (shares HLA-A1, ◇), VMM15 (shares HLA-A1, □), VMM17 (shares HLA-A*0201, △), or HT144 (shares HLA-A1, ×). **B** They were assayed also on a panel of HLA-A*0201$^+$ melanomas in a similar fashion. There was some non-specific background reactivity at the highest E:T ratio, even to the negative control DM14 (△), but the only significant CTL reactivity was to DM331 tumor cells (●). Tumors not recognized by these CTL, but that share HLA-A1 or HLA-A*0201 with the autologous tumor, included A375 (shares HLA-A*0201, ■), SkMel24 (shares HLA-A1, A*0201, ◇), Bowes (shares HLA-A*0201, ○), DM14.A2-17 (shares HLA-A*0201, ▲), DM93 (shares HLA-A*0201, −), and DM6 (shares HLA-A*0201, ×). **C** DM331 CTL were cultured as above and assayed on day 38 of culture at an E:T ratio of 10:1 on DM331 tumor cells that had been preincubated with w6/32, an antibody to a monomorphic determinant of class I MHC molecules (○), or L243, an antibody to a monomorphic determinant on HLA-DR molecules (■)

DM331 was observed both with peptides from HLA-A1, pulsed on the HLA-A1$^+$ lymphoblastoid cell C1R-A1 (Fig. 6A), and with peptides from HLA-A*0201 pulsed on the HLA-A2$^+$ cell T2 (Fig. 6B). When those same naturally processed peptides were evaluated for their ability to reconstitute CTL epitopes for VMM15 CTL (restricted by HLA-A1) or for VMM5 CTL (restricted by HLA-A*0201), no reactivity was observed (data not shown). Thus at least two peptide epitopes for DM331 CTL are restricted by HLA-Al, and at least one is restricted by HLA-A*0201.

The identity of the peptide epitopes recognized by DM331 CTL is being sought. DM331 and three other melanomas were screened by PCR for expression of MAGE-1, MAGE-2, MAGE-3, BAGE, GAGE, MART-1/Melan-A, Tyrosinase, gp100, and p15. All of these antigens are expressed by DM6 and by DM93 melanomas, but neither of those tumors is recognized by DM331 CTL. Only MAGE-3 and p15 were expressed by



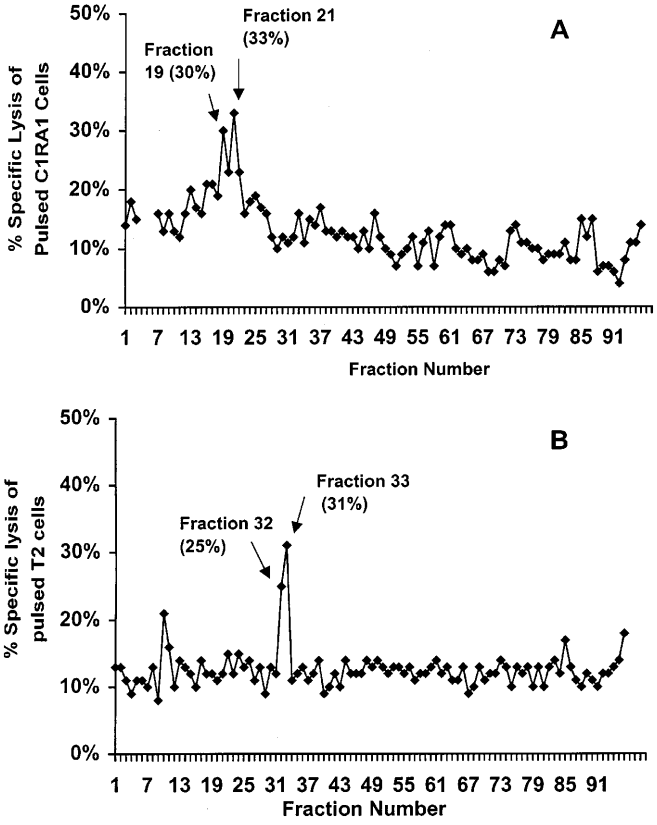


Fig. 6A, B Naturally processed peptides eluted from HLA-A1 and HLA-A*0201 molecules of DM331 melanoma cells were evaluated for their ability to reconstitute CTL epitopes for DM331 CTL. HLA-A1-associated peptides (**A**) and HLA-A*0201-associated peptides (**B**) from melanoma DM331 were isolated by immunoaffinity purification and fractionated using rpHPLC. **A** The 96 HPLC fractions of naturally processed HLA-A1-associated peptides were pulsed on C1R-A1 cells (**A**), and the 96 HPLC fractions of naturally processed HLA-A*0201-associated peptides were pulsed on T2 cells (**B**), which were then evaluated for susceptibility to autologous (DM331) CTL in 4-h ^{51}Cr-release assays. For each graph, the average lysis from two assays is represented. The mean DM331 tumor lysis was 53%. Lysis of T2 cells without peptides was 13%. In **A**, fractions 19 and 21 consistently were associated with lysis 10%–20% above background. In **B**, fractions 32 and 33 consistently demonstrated lysis 10%–20% above background

Table 3 Expression of defined melanoma antigens, determined by the polymerase chain reaction

Antigen	DM6	DM14	DM93	DM331
MAGE-1	++	+	+	–
MAGE-2	+	–	+	–
MAGE-3	++	+	++	+
BAGE	+	–	+	–
GAGE	++	+	++	–
MART-1/Melan-A	++	–	++	–
Tyrosinase	++	–	++	–
Gp100	+	–	++	–
P15	+	+	+	+

DM331 (Table 3). Thus, none of these shared antigens can explain the specific reactivity of DM331 CTL for autologous tumor cells.

Discussion

In this study, we initially characterized four HLA-A*0201$^+$ melanomas that fail to be lysed by murine and human CTL reactive against multiple shared melanoma antigens. The reactivity of the CTL tested is broad, with defined epitopes including, but not limited to, MART-$1_{27–35}$ (AAGIGILTV), Pmel17/gp$100_{280–288}$ (YLEPGPVTA), Pmel17/gp$100_{154–162}$ (KTWGQYWQV), and tyrosinase$_{369–377}$ (YMDGTMSQV). We found that the failure of these melanomas to be lysed by CTL is due to their failure to express proteins that give rise to shared CTL epitopes. In particular, they lack expression of four different MDP, including tyrosinase, Pmel17/gp100, TRP-1/gp75, and MART-1/Melan-A, all of which are known sources of shared MHC-restricted CTL epitopes. The phenotype of MDP loss observed in the four well-characterized melanoma lines A375, DM14.A2-17, DM331, and SkMel24 was also evident in several other melanoma cell lines that were not studied in as much detail, including VMM17, Na8Mel and Bowes. Thus, this phenotype may well be quite common.

Chen et al. reported that expression of tyrosinase, gp75, and gp100 in 16 surgical specimens of melanoma was discordant [8]. However, the immunohistochemical data included in that report do reveal that 4 of the 16 specimens studied (25%) lacked all three of those MDP. HMB45 staining is often heterogeneous [8]; however, it is significant that, in the 16 melanomas studied by Chen, all those negative for gp100 expression were also negative for tyrosinase and gp75 expression, as determined by immunohistochemistry [8]. That study did not correlate CTL reactivity with expression of MDP, and it did not evaluate MART-1/Melan-A expression, but its findings are consistent with the observations of the present manuscript, that a distinct phenotype of melanoma cells exists that is MDP-negative, which is relatively common. A question can be raised whether the tumor lines studied in detail in this manuscript lost expression of MDPs in vivo or whether the antigen-loss phenotype is the result of in vitro artefact. The melanomas from which the DM14 and DM331 cell lines were derived were evaluated by immunohistochemistry and by standard microscopy. They were both amelanotic, and expression of gp100 was limited to 5%–15% of the melanoma cells. Thus, both of these tumor lines are phenotypically consistent with the majority of the tumor cells in vivo. Information on the original tumor for SKMel24 is not available, but the A375 line is reported to grow in vivo, in nude mice, as an amelanotic nodule [17]. Thus, there is direct evidence, for at least three of the four lines, that the absence of MDP expression is not simply an in vitro artefact.

The mechanism for concordant loss of MDP expression in the melanoma cell lines studied is not known. However, the genes encoding tyrosinase, Pmel17/gp100, and gp75 are all located on different chromosomes; thus, the failure to express all these MDP seems unlikely to be due to independent deletions or mutations of each of these genes. Instead, it seems likely to be the result of common transcriptional control. The control of transcription of tyrosinase and other MDP is complex and remains incompletely understood. However, it has been shown that transfection of murine melanocytes with v-*ras*^{Ha} results in transformation to an amelanotic phenotype, characterized by the concordant loss of expression of tyrosinase, TRP-1, TRP-2, and the silver locus, while several other melanosomal proteins are sharply down-regulated, and typical melanosomes are absent [14]. Thus, there is evidence for concordant transcriptional control of MDP expression in murine melanocytes, which can be disrupted by oncogenic transformation. We postulate that the same may well be true for human melanocytes and melanoma. The association of the MDP-negative phenotype with the oncogene v-*ras*^{Ha} raises the possibility that a stepwise progression of melanoma may include alterations in transcriptional control of the melanocytic differentiation proteins. If the mechanism for this change can be identified, it may offer insight into the progression of the malignant phenotype and it might also suggest therapeutic approaches for these MDP-negative tumors.

Despite the absence of known shared melanoma antigens presented by HLA-A*0201 on the four MDP-negative melanomas, all the melanomas studied retained other features required for recognition and lysis by CTL. Specifically, there was no down-regulation of HLA-A*0201 expression, TAP1 expression was normal, and both TAP-dependent and TAP-independent antigen-processing pathways were intact. In addition, these cells remain susceptible to lysis by murine and human CTL when CTL epitopes are reconstituted by pulsing with appropriate peptides. Thus, it is reasonable to suspect that loss of MDP protein expression, while conferring resistance to lysis by allogeneic CTL, may be insufficient for complete immune escape in the autologous setting, as long as additional tumor antigens may be expressed.

At least one of these tumors (DM331) does, in fact, express antigens recognized by autologous T-cells. These T cells do not recognize any of a large panel of shared HLA-A*0201-restricted or HLA-A1-restricted melanoma antigens, as shown by their failure to lyse multiple HLA-A*0201$^+$ or HLA-A1$^+$ melanomas that express these antigens. They also fail to recognize other MDP-negative melanomas. Rather, DM331 CTL appear to recognize unique or undefined antigens restricted by HLA-A*0201 and by HLA-A1.

It is not possible to state with certainty that the favorable clinical course observed in this patient DM331 is related to the T cell response observed in vitro. However, the fact that circulating T cells in this patient can recognize autologous-cell-specific antigens may well have had an impact in this patient's good clinical outcome to date. Furthermore, these data provide direct

evidence that multiple MHC-associated epitopes expressed on melanoma cells can permit immune recognition of tumor cells that have lost expression of many defined shared antigens. Thus, immunotherapy directed against shared melanoma antigens may be improved by supplementing with immunotherapy directed against unique or undefined autologous antigens, especially in those patients whose tumors do not express melanocytic differentiation proteins. This may be possible by using autologous tumor cells, or autologous-tumor-derived mRNA [23] as an antigen source. Immunotherapy directed against shared antigens at least should be evaluated in the context of the antigenic profile of the patient's tumor.

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