Melanoma Cells and Normal Melanocytes Share Antigens Recognized by HLA-A2–restricted Cytotoxic T Cell Clones from Melanoma Patients

By Andrea Anichini, Cristina Maccalli, Roberta Mortarini, Stefania Salvi, Arabella Mazzocchi, Paola Squarcina, Meenhard Herlyn,* and Giorgio Parmiani

*From the Division of Experimental Oncology D, Istituto Nazionale Tumori, 20133 Milan, Italy, and *The Wistar Institute, Philadelphia, Pennsylvania 19104*

Summary

HLA-A2–restricted, $CD3^+$, $CD8^+$, α/β^+ cytotoxic T cell (CTL) clones were isolated from peripheral blood (PBL) or tumor infiltrating lymphocytes (TIL) of two $HLA\text{-}A2^+$ melanoma patients (9742 and 5810), to evaluate the possible recognition of autologous melanoma and of allogeneic HLA-A2–matched normal melanocytes. These CTL clones lysed not only fresh and cultured autologous melanoma cells, but also allogeneic $HLA\text{-}A2^+$, but not $HLA\text{-}A2^-$, normal melanocytes. The lysis of autologous neoplastic cells and of melanocytes could be inhibited by an anti-HLA-A2 monoclonal antibody (mAb). Lysis of the normal melanocytes was not dependent on the presence of human or fetal calf serum in the culture medium. HLA-A2–restricted CTL clones recognized not only proliferating melanocytes cultured in complete melanocyte medium, but also melanocytes made quiescent by culture for up to 6 d in a basal medium devoid of exogenous factors such as phorbol ester (*O*-tetradecanoyl phorbol 13-acetate [TPA]), epidermal growth factor, insulin, and pituitary extracts. Analysis of specificity of four CTL clones (A75, A83, A94, and 119) from patient 9742, performed on a panel of 39 targets, indicated that the three HLA-A2–restricted CTL (A75, A83, and A94) lysed all but one of nine allogeneic melanomas expressing the HLA-A2 molecule with no reactivity on nine $HLA\text{-}A2^-$ allogeneic melanomas. Only a few instances of borderline reactivity were seen by the same effectors on 21 targets of nonmelanocyte lineage, including 12 carcinomas of different histology, four Epstein-Barr virus-transformed B cells (lymphoblastoid cell lines [LCL]), including the autologous LCL, four lines of normal fibroblasts, and normal kidney cells. Lack of reactivity on allogeneic targets of nonmelanocyte lineage occurred in spite of expression of HLA-A2 on 14 of these targets as determined by conventional tissue typing and cytofluorimetric analysis with four different anti-HLA-A2 mAb. These data indicate that tissue-related antigens can be expressed on normal and neoplastic cells of the melanocyte lineage and can be recognized in association with HLA-A2 by CTL clones from melanoma patients.

The clonal analysis of T cell–mediated response to human melanoma has indicated that some of the CTL clones from a number of patients can recognize tumor-associated antigens (TAA)[1] by a TCR-dependent and HLA-restricted mechanism (1). These CTL clones are thought to recognize TAA by the same mechanism that controls T cell recognition of conventional antigens, i.e., as peptides associated with one of the HLA molecules expressed by the patient's neoplastic cells (2).

At least two hypotheses have been put forward to explain the genesis of the antigenic peptides seen as TAA by autologous CTL clones. The first one is that antigenic peptides may originate from the intracellular processing of proteins whose genes should be expressed in neoplastic but not in normal cells. Molecular evidence in agreement with this mechanism has been recently obtained for an antigen recognized on melanoma cells in association with HLA-A1 (3). A second possibility is that the generation of antigenic peptides in human tumors is dependent on the presence in the neoplastic cells of gene mutations affecting the structure of proteins expressed also in normal cells. Evidence consistent with this mecha-

[1] *Abbreviations used in this paper:* ADAbp, adenosine deaminase binding protein; EGF, epidermal growth factor; ICAM-1, intercellular adhesion molecule; LCL, lymphoblastoid cell line; MCM, melanocyte complete medium; MLTC, mixed lymphocyte tumor culture; mCSP, melanoma chondroitin sulfate proteoglycan; PHS, pooled human serum; TAA, tumor-associated antigen; TIL, tumor-infiltrating lymphocyte.

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nism has been obtained by analysis of the antigenicity of peptides corresponding to regions of RAS oncogenes altered by point mutations (4). Both models of TAA generation share the assumption that antigens seen by human T lymphocytes on neoplastic cells should not be expressed on the normal cells of the same tissue (5). However, this hypothesis could not be verified in melanoma because of the lack of normal cells sharing the appropriate HLA-restricting element and the same histological origin with the neoplastic cells recognized by tumor-specific and HLA-restricted CTL clones.

In this study, CTL clones from melanoma patients were tested for cytotoxic activity not only on autologous tumor but also on allogeneic HLA-A2–matched melanocytes. The results indicate that among the PBL or tumor-infiltrating lymphocytes (TIL) of two HLA-A2$^+$ melanoma patients, one with primary and the other with metastatic disease, it is possible to find some CD3$^+$, CD8$^+$, α/β^+ CTL clones that lyse autologous and allogeneic HLA-A2$^+$ melanoma cells and allogeneic HLA-A2$^+$ normal melanocytes. These data show for the first time that tissue-related antigens can be expressed on normal and transformed cells of the melanocyte lineage and be recognized in an HLA-restricted fashion by patients' T lymphocytes.

Materials and Methods

Normal and Neoplastic Cells. All melanoma cells used in this study were isolated from primary and metastatic lesions obtained from surgical specimens of patients admitted for surgery to the Istituto Nazionale Tumori in Milan. All tumor and normal cells were kept in culture with 10% FCS-RPMI-1640. Melanoma cells to be used as stimulators for mixed lymphocyte tumor cultures (MLTC) or for T cell clones and all targets to be used in cytotoxic assays were instead cultured in 10% pooled human serum (PHS)-RPMI-1640. Normal human melanocytes (FM 216/A, FM713, FM727, and FM741) isolated from neonatal foreskin were cultured with a chemically defined medium as described (6). Briefly, the melanocyte complete medium (MCM) consisted of a base medium with four parts of MCDB 153 medium supplemented with 2 mM Ca^{2+} and one part of Leibovitz's L15 medium, 5 ng/ml epidermal growth factor (EGF), 40 μg/ml bovine pituitary extract, 5 μg/ml insulin, 10 ng/ml phorbol ester (TPA) (all from Sigma Immunochemicals, St. Louis, MO), and 2% FCS (ICN Biomedicals, Hyland, CA) or 2% PHS. For some experiments MCM was replaced with a basal medium lacking EGF, pituitary extract, insulin, and TPA. Normal and neoplastic cells were also checked by electron microscopy for the absence of mycoplasma contamination and for the presence of premelanosomes in cells of the melanocyte lineage. EBV-transformed lymphoblastoid cell line [LCL] from four melanoma patients (9742, 10538, 1811, and 4405) were produced by infection of PBL with supernatant from the EBV producer line B95.8 as described (7).

T Cell Clones. PBL and TIL were isolated as described (7) from melanoma patients 9742 (HLA-A2, -A24; -B13, -B18; -Cw6, -Cw7; -DR7, -DR11; and -DQ4) and 5810 (HLA-A2, -A24; -B18, -B63; -Cw7; -DR6, -DR11; and -DQ1, -DQ7). The lymphocytes (10^6/ml) were cultured for 3–4 wk with autologous irradiated (10,000 rad) metastatic (patient 9742) or primary (patient 5810) melanoma cells first in 24-well plates (model 3424; Costar Corp., Cambridge, MA) and then in 25 cm^2 flasks (model 25100B; Bibby Scientific Products Ltd., Stone, UK) at a lymphocyte/tumor ratio of 5:1 in 10% PHS-RPMI-1640 in the presence of 50 U/ml of rIL-2 (EuroCetus, Amsterdam, The Netherlands). The cultures were restimulated weekly with autologous tumor cells. At the end of 4 wk of culture, cells were cloned by limiting dilution. Cloning was performed as described (7) in 96 round-bottomed well plates (model 3799; Costar Corp.) with 10% PHS-RPMI-1640 supplemented with 50 U/ml of rIL-2 in the presence of irradiated autologous tumor cells (5×10^3/well) and allogeneic pooled lymphocytes (5×10^4/well, irradiated at 3,000 rad) from at least three donors as a source of feeder cells. Cloning was performed at 5, 1, 0.5, and 0.25 cells/well. The plates were restimulated with autologous tumor cells and feeder cells after 1 wk of culture and screened at day 14 for the presence of growing clones. On the basis of the cloning efficiency, which ranged between 0.5 and 17%, a frequency of clonogenic precursors was evaluated and used to determine the probability of clonality of each clone by Poisson statistics as described by Taswell et al. (8). Only clones having a p value for true clonality of at least 0.95 were used in this study. True clonality of the effectors used in this study was confirmed by analysis of TCR-Vα and -Vβ expression in each clone, performed by RT-PCR with primers specific for all known members of the Vα and Vβ gene families. This analysis revealed that only one Vα and one Vβ mRNA was exressed by each clone (Sensi, M., S. Salvi, C. Castelli, C. Maccalli, A. Mazzocchi, R. Mortarini, M. Herlyn, G. Permiani, and A. Anichini, manuscript in preparation). 156 clones from patient 9742 and 223 clones from patient 5810, selected on the basis of a p value for clonality >0.95, were screened for cytotoxicity on the autologous melanoma and, at the same time, for inhibition of tumor lysis by anti-CD3 and anti-HLA class I antibodies. 63 clones from patient 9742 (30 from TIL and 33 from PBL) and 28 clones from patient 5810 (12 clones from TIL and 16 from PBL) expressed significant cytotoxic activity on the autologous tumor, and were inhibited by both anti-CD3 and anti-HLA class I mAb. CTL clones from patient 9742 were also obtained by direct cloning at 0.5 and 0.25 cells/well of fresh PBL in the presence of allogeneic irradiated PBL, 50 U/ml of rIL-2, and 1% PHA (Difco Laboratories, Inc., Detroit, MI). 207 clones with a p value for clonality of 0.96 were screened for cytotoxic activity and 21 were cytotoxic on the autologous tumor and inhibited by anti-HLA class I mAb. All CTL clones used in this study expressed a CD3$^+$, CD4$^-$, CD8$^+$, α/β^+ phenotype as assessed, respectively, with antibodies OKT3 (CRL8001; American Type Culture Collection [ATCC], Rockville, MD), OKT4 (CRL8002; ATCC), OKT8 (CRL8014; ATCC) and by the anti-TCR-α/β mAb WT31 (Becton Dickinson & Co., Mountain View, CA).

Cytotoxicity Assay. Target cells (10^6) were labeled for 90 min at 37°C with 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham International, Amersham, Bucks, UK). After three washings with cold medium, the targets were adjusted to 10^4/ml in 10% PHS-RPMI-1640. Effectors were resuspended at various concentrations to give final effector/target ratios ranging between 50:1 and 5:1. Effectors and targets were then seeded in 0.1 ml aliquots in v-bottomed 96-well plates (model 3596; Costar Corp.), centrifuged at 60 g for 5 min and incubated at 37°C, 5% CO_2 for 4 h. At the end of the incubation time, 0.125 ml of supernatant was collected, mixed with 0.7 ml of cocktail (Ready-Solve HP 158726; Beckman Instruments, Geneva, Switzerland) in Micronic PPN tubes (Flow Laboratories, Settimo Milanese, Italy) and counted in a beta counter (model LS1801; Beckman Instruments). Results (in cpm) were expressed as follows: percent lysis = experimental release − spontaneous release/maximum release − spontaneous release, where spontaneous release was assessed by incubating target cells in the absence of effectors and maximum release was determined in the presence of

1% NP-40 detergent (BDH Biochemicals, Poole, UK). Inhibition of lysis by mAb to CD3, HLA-A2, HLA class I, and HLA-DR antigens was performed by preincubating the effectors or the targets with different purified mAb in 0.1 ml for 45 min at 37°C. Then effectors or targets were added in 0.1 ml aliquots and the cytotoxicity assay was carried out as described above. The assays were performed at the final concentration of 1 μg/ml for each mAb.

Proliferation Assay. Melanocytes were incubated for 48–72 h in 0.2 ml of MCM or in a basal medium without EGF, pituitary extracts, insulin, and TPA in flat-bottomed 96-well plates (model 3595; Costar Corp.) at the initial concentration of 2×10^4 cells/well. The cultures were pulsed with 1 μCi [^{3}H]thymidine/well (sp act. 6.7 Ci/mmol) (Amersham International) during the last 8 h of cultures. Each sample was then harvested, absorbed onto nitrocellulose paper, and washed using a harvester (Titertek; Flow Laboratories). The nitrocellulose filters were dried and counted after liquid scintillation in a beta counter (model LS1801; Beckman Instruments). The proliferation assay was performed in six replicate wells.

mAb and Phenotypic Analysis. Phenotype of CTL clones, melanocytes, and all tumor cells was performed by indirect immunofluorescence followed by cytofluorimetric analysis on a FACScan® cytofluorimeter (Becton Dickinson & Co.) as previously described (9). The following mAb were used: W6/32 (10), anti-HLA class I; CR11.351 (11), BB7.2 (12), PA2.1 (13) and MA2.1 (14), anti-HLA-A2; R24 (15), antiganglioside GD3; 528 (16), anti-EGF-receptor; 17F11 (17), anti-c-*kit* protein; C350 (18), anti-gp180; B5.2 (18), anti-melanoma chondroitin sulfate proteoglycan (mCSP); S27 (19), anti-adenosine deaminase binding protein (ADAbp); ME20.4 (6), anti-nerve growth factor (NGF) receptor; ME9–61 (6), anti-p97 melanotransferrin; TA99 (20), anti-gp75 tyrosinase-related glycoprotein; TR66 (21), anti-CD3; 84H10 (22), anti-intercellular adhesion molecule 1 (ICAM-1); and TS2/9 (23) anti-LFA-3.

Results

Lysis of Autologous HLA-A2$^+$ Tumor and Allogeneic HLA-A2$^+$ Melanocytes by CTL Clones from TIL and PBL of Melanoma Patients. Table 1 shows the biological characterization of the human newborn foreskin melanocytes used in this study. All melanocytes are pigmented cells; have a bipolar/spindle morphology; grow as anchorage-dependent cells; are not tumorigenic when injected into nude mice; have a normal diploid karyotype, undergo senescence after a limited number of passages in vitro; and three out of four depended on the presence of phorbol ester for growth. By cytofluorimetric analysis, all these cells express HLA class I antigens as detected by the antibody w6/32 (10). In addition, melanocytes FM741 and FM727 are positive for HLA-A2 after staining with the anti-HLA-A2 antibody CR11.351 (11), whereas the melanocytes FM216/A and FM713 do not react with the mAb. The expression of HLA-A2 on melanocytes FM741 and FM727 and the lack of this determinant on FM713 and FM216/A was confirmed by conventional HLA tissue typing and by immunofluorescence with three additional anti-HLA-A2 mAb, MA2.1 (13), BB7.2 (12), and PA2.1 (14) (data not shown). All other phenotypic markers were similarly expressed on the four melanocyte cultures. In fact, as described for proliferating melanocytes in vitro (6, 24), all melanocytes expressed the ganglioside GD3 (15) and a low but significant amount of the adhesion molecule ICAM-1 (22). All melanocytes reacted significantly with an anti-LFA-3 antibody (23). In addition, all melanocytes were weakly positive for the EGF receptor (16) and for mCSP, which are considered early markers of melanocyte differentiation (18), but were strongly positive for gp180, recognized by antibody C350, a late marker of melanocyte differentiation (18). Also, all cells expressed the tyrosinase-related gp75 marker (20), the NGF receptor (6), and p97, melanotransferrin (6). Furthermore, all melanocytes expressed the product of the *c-kit* gene (17) and ADAbp (19), two antigens that are expressed on normal melanocytes but that are lost on melanoma cell lines (19, 25).

These melanocytes were used as targets to screen CTL clones from two HLA-A2$^+$ melanoma patients. CTL clones with cytolytic activity on melanocytes were isolated from a larger panel of 112 CTL clones initially selected for: (*a*) cytotoxic activity on the autologous tumor; and (*b*) blocking of melanoma lysis by anti-CD3 and anti-HLA class I mAb. Table 2 shows that some of these effectors, sharing a CD3$^+$ CD8$^+$ WT31$^+$ phenotype and isolated from PBL or TIL of two HLA-A2$^+$ melanoma patients, could lyse not only the autologous tumor but also some allogeneic melanocytes. Lysis of autologous tumor by CTL clones A75, A81, A83, A94, A103, A37, A54, and 211, but not 119, could be inhibited by preincubating melanoma cells with the anti-HLA-A2 mAb CR11.351. This is in agreement with the known blocking activity of this mAb. In fact, a previous report from this laboratory has shown that antibody CR11.351 can block the lysis of any HLA-A2$^+$ target by an alloreactive HLA-A2–specific CTL clone (26). In addition, only allogeneic melanocytes expressing HLA-A2 (FM741 and FM727) were recognized by the same effectors, suggesting an HLA-A2–restricted recognition of antigens expressed on autologous melanoma and allogeneic normal melanocytes. Clone 119 represents an example of the many CTL clones isolated from the two patients that did not recognize allogeneic HLA-A2$^+$ melanocytes and were not blocked by mAb CR11.351 in the lysis on the autologous tumor, thus indicating that clones such as 119 are not HLA-A2 restricted.

Cytolytic Activity on Normal Melanocytes by CTL Clones Is HLA-A2 Restricted and Not Dependent on the Proliferative Status of the Target Cells or on Composition of the Melanocyte Culture Medium. Table 3 shows that the lysis of HLA-A2$^+$ melanocytes by two different CTL clones (CTL A83 and CTL A94) from patient 9742 could be inhibited by preincubating the targets with the anti-HLA-A2 mAb CR11.351, indicating that the recognition of these cells occurs by the same mechanism seen for the lysis of the autologous melanoma. Also, lysis of melanocytes was not due to the presence of bovine or human serum in the culture medium. In fact, HLA-A2$^+$ melanocytes were equally lysed after culture for at least 2 wk in the presence of either 2% FCS or 2% PHS, whereas one HLA-A2$^-$ melanocyte was not recognized after culture in the presence of either FCS or PHS.

We next tested the possibility that the proliferative status of the melanocytes might influence antigen recognition by CTL clones. In fact, and in agreement with the data of Table 1, it is known that proliferating human melanocytes in culture can undergo phenotypic changes characterized by de novo

Table 1. *Characterization of Human Foreskin Melanocytes*

Characteristic		Melanocytes			
		FM741	FM727	FM713	FM216/A
Morphology		Bipolar	Bi- to tripolar	Tripolar	Spindle
Pigmentation (+ to + + + +)		+	+ +	+ + +	+ +
Karyotype		Diploid	ND	Diploid	Diploid
Growth in soft agar (% colony forming efficiency)		<0.001%	<0.001%	<0.001%	<0.001%
Tumorigenicity (No. of mice with tumors/total injected)		0/4	ND	ND	0/4
Dependence on phorbol ester		Yes	Yes	Yes	No
Maximum No. of passages before senescence		31	21	39	29
Phenotype	*mAb*				
HLA-I*	w6/32	+ + + +‡	+ + + +	+ + + +	+ + + +
HLA-A2	CR11.351	+ + + +	+ + + +	−	−
ICAM-1	84H10	+	+	+	+
LFA-3	TS2/9	+ +	+ +	+ +	+ +
GD3 Ganglioside	R24	+ + +	+ + +	+ + +	+ + +
EGF Receptor	528	+	+	+	+
gp180	C350	+ + + +	+ + + +	+ + + +	+ + + +
mCSP	B5.2	+	+	+	+
c-Kit protein	17F11	+ +	+ +	+ +	+ +
gp75 (Tyrosinase-related)	TA99	+ + + +	+ + + +	+ + + +	+ + + +
NGF-Receptor	ME20.4	+ + + +	+ + + +	+ + + +	+ + + +
p97 Melanotransferrin	ME9-61	+ + + +	+ + + +	+ + + +	+ + + +
ADAbp	S27	+ +	+ +	+ +	+ +

All melanocytes (FM741, FM727, FM713, and FM216/A) grow in liquid culture as adherent cells but do not grow in semisolid medium (0.35% agarose). Tumorigenicity was tested by subcutaneous injection of 10^6 cells into nude mice.

* Phenotype of proliferating melanocytes kept in MCM was evaluated by indirect immunofluorescence with the indicated antibodies. Expression of gp75 was measured on permeabilized cells. Expression of HLA-A2 on melanocytes was tested with identical results also with antibodies PA2.1 (13), BB7.2 (12), and MA2.1 (14).

‡ Results are expressed as follows: −, <10% positive cells; +, 11–25% positive cells; + +, 26–50% positive cells; + + +, 51–80% positive cells; + + + +, >80% positive cells.

expression of serologically defined antigens normally found on melanoma but not on resting melanocytes (6, 24) such as the ganglioside GD3 and ICAM-1. However, antigenic recognition of melanocytes by CTL clones was seen not only on proliferating cells, as when using target melanocytes cultured in MCM, but also on growth-arrested cells. In fact (Table 3), HLA-A2$^+$ quiescent melanocytes, obtained by culture for up to 6 d in a basal medium devoid of all main exogenous factors (EGF, insulin, TPA, and pituitary extracts), were still recognized, even though with less efficiency, by CTL clones A83 and A94. However, the quiescent melanocytes were recognized with lower efficiency also by LAK cells, the positive control of lysability. A possible reason for the reduced lysability of resting melanocytes was found, through FACS® analysis, by comparing the phenotype of proliferating and quiescent cells. In fact, HLA class I antigens and HLA-A2 molecules were expressed at similar levels, whereas ICAM-1 was absent on resting cells (data not shown).

In addition, as seen for proliferating melanocytes, the lysability of growth-arrested melanocytes was not influenced by presence of FCS or of PHS in the basal medium (data not shown). Test of lysis with quiescent melanocytes maintained in basal medium for more than 6 d could not be performed because substantial cell death ensued after this time point.

Table 2. *Lysis of Autologous Melanoma and of Allogeneic Melanocytes by CTL Clones from TIL and PBL of Melanoma Patients*

Patient	Effector	Lysis of autologous melanoma		Lysis of melanocytes			
		− mAb‡	+ mAb‡	FM216/A HLA-A2⁻	FM713 HLA-A2⁻	FM727 HLA-A2⁺	FM741 HLA-A2⁺
9742							
	PBL A75*	45§	9‖	0	0	31	22
	PBL A81	33	6‖	1	0	40	27
	PBL A83	65	21‖	0	4	39	30
	PBL A94	45	7‖	0	0	30	25
	PBL A103	28	1‖	0	0	25	14
	PBL 119	32	31	0	0	ND	1
	TIL A37	20	0‖	0	2	20	8
	TIL A54	24	0‖	1	2	33	16
	LAK	50	50	22	49	34	41
5810							
	PBL 211	18	0‖	0	0	26	10
	LAK	54	51	43	31	28	31

Lysis of autologous tumor (Me9742 or Me5810) and of allogeneic melanocytes by CTL clones isolated from TIL or PBL of two melanoma patients was tested in a 4-h ^{51}Cr-release assay at the E/T ratio of 10:1.

* CTL clones A75, A81, A83, A94, A103, 211 (isolated from PBL), A37, and A54 (isolated from TIL) were obtained after MLTC, whereas clone 119 (isolated from PBL) was obtained by direct cloning of fresh PBL in the presence of 1% PHA. LAK cells were produced by culturing allogeneic PBL from a normal donor in the presence of 100 U/ml of rIL-2 for 1 wk.

‡ Lysis of Me9742 and of Me5810 was tested with or without preincubating target cells with 1 μg/ml of the anti-HLA-A2 mAb CR11.351.

§ Results expressed as percent lysis. Underlined values represent significant values of lysis in comparison to the spontaneous release of each target (SNK test $p = 0.01$).

‖ Lysis of the target in the presence of mAb is significantly different from lysis of the same target in the absence of mAb (SNK test, $p = 0.01$).

These data indicate that some antimelanoma CTL clones can react in an HLA-A2–dependent fashion to antigens expressed on proliferating and resting normal melanocytes.

HLA-A2–restricted CTL Clones Lyse only HLA-A2⁺ Cells of the Melanocyte Lineage. Table 4 shows the analysis of specificity of three of the CTL clones from patient 9742 that lysed allogeneic HLA-A2⁺ melanocytes (CTL A75, CTL A83, and CTL A94) and of one CTL clone that did not kill these cells (CTL 119). As expected, on the basis of the selection criteria adopted to isolate these clones, the lysis of autologous tumor by all clones, but not by control LAK cells, was inhibited by two different anti-CD3 antibodies (OKT3 and TR66) and by the anti-HLA class I mAb W6/32, but not by a control anti-HLA-DR mAb (L243) (27). CTL clones A75, A83, and A94 lysed all but one (Me4855) allogeneic melanomas expressing HLA-A2, but did not lyse the melanomas lacking this determinant, with the exception of a borderline reactivity by CTL clone A83 on melanomas 665/1 and 4405. In addition, all four CTL clones failed to lyse the two HLA-A2⁺ LCL obtained by EBV infection of PBL from the HLA-A2⁺ patients 9742 (i.e., the autologous LCL) and 10538. All clones expressed borderline or no lytic activity on most of the allogeneic and normal cells of nonmelanocyte lineage, including 12 carcinomas of different histology, four lines of normal fibroblasts (F1338/1, F8536, F9203, F3046), and normal kidney cells (Re458), even though HLA-A2 was expressed on 10 of these targets. Only one of the HLA-A2–restricted clones (A83) expressed a low but significant lytic activity on two HLA-A2⁺ fibroblasts (F1338/1 and F3046) and on one HLA-A2⁺ renal carcinoma (Reca458).

The specificity of CTL clone 119 indicated that additional antigenic determinants, seen in association with HLA class I alleles which are different from HLA-A2, must be expressed on melanoma 9742. In fact, Table 4 shows that the cytotoxic activity of this clone on the autologous tumor is inhibited by antibodies to CD3 and to HLA class I antigens, indicating that this clone acts by a TCR-dependent and HLA class I–restricted mechanism. Lack of lysis of allogeneic targets, either in the melanocyte or in the nonmelanocyte lineage by CTL 119 might be dependent on the presence of unique antigens seen only on Me9742 cells or on the lack of appropriate restriction element on the allogeneic targets. The latter possibility is in agreement with the observation made by comparing the HLA phenotype of the tumor 9742 with that of the allogeneic targets used for the specificity assay (data not shown). This comparison revealed the existence of a few

Table 3. *HLA-A2-restricted Lysis of Proliferating and Resting Melanocytes by CTL Clones*

Melanocytes	Medium	Anti-HLA-A2 mAb	Percent lysis		
			CTL A83	CTL A94	LAK
HLA-A2$^+$					
FM727	MCM + 2% PHS*	−‡	49§	39	58
FM727	MCM + 2% PHS	+	12‖	0‖	55
FM727	MCM + 2% FCS	−	47	33	53
FM727	Basal + 2% FCS (72 h)	−	28	21	30
FM727	Basal + 2% FCS (144 h)	−	26	20	37
FM741	MCM + 2% PHS	−	44	39	56
FM741	MCM + 2% PHS	+	4‖	0‖	55
FM741	MCM + 2% FCS	−	37	26	55
FM741	Basal + 2% FCS (72 h)	−	19	17	25
FM741	Basal + 2% FCS (144 h)	−	26	20	39
HLA-A2$^-$					
FM713	MCM + 2% PHS	−	0	0	38
	MCM + 2% FCS	−	0	0	36

Lysis of melanocytes was tested in a 4-h ^{51}Cr-release assay at the E/T ratio of 10:1. CTL A83 and A94 derived from MLTC of PBL from Patient 9742. LAK cells were produced as described in the legend to Table 2.

* Melanocytes were cultured in MCM as described in Materials and Methods in the presence of either 2% FCS or 2% PHS for at least 2 wk before using them in the cytolytic assay. The basal medium consisted of MCDB 153 plus L15 without EGF, insulin, pituitary extract, and TPA. After 48 h of culture in MCM the [^{3}H]thymidine incorporation by FM727 and FM741 was 6,603 ± 522 cpm and 5,887 ± 634 cpm, respectively. [^{3}H]Thymidine incorporation of the same cells after 48 h of culture in basal medium was 467 ± 201 cpm (FM727) and 672 ± 310 cpm (FM741). [^{3}H]Thymidine incorporation of FM727 and FM741 became negligible after 72 h of culture in basal medium and the cells began to die progressively after 144 h.

‡ Lysis of melanocytes was inhibited by preincubating the targets with 1 μg/ml of the mAb CR11.351.

§ Results expressed as percent lysis. Underlined values represent significant values of cytotoxicity in comparison to the spontaneous release of each target (SNK test, $p = 0.01$).

‖ Lysis in the presence of the antibody is significantly different from lysis in the absence of antibody (SNK test, $p = 0.01$).

matches for HLA-A2, -A24, -B18, -Cw6, and -Cw7, but no matches were available for the HLA class I allele B13 expressed by 9742 cells.

Finally, all clones shown in Table 4 lysed not only the cultured tumor, but also the fresh, uncultured melanoma, even though to a lesser extent than the cultured tumor. However, the fresh tumor was recognized with lower efficiency than the cultured tumor also by LAK cells used as positive control of target lysability. By cytofluorimetric analysis, the fresh tumor expressed, in comparison with the cultured tumor, lower amounts of HLA class I, HLA-A2 antigens, and of two main adhesion molecules, ICAM-1 and LFA-3 (data not shown). The reduced expression of these critical determinants of the effector–target interaction may explain the reduced lysability of the fresh tumor.

Taken together, the results of the specificity analysis indicate that CTL clones such as A75, A83, and A94 recognize, in a TCR-dependent and HLA class I–restricted fashion, antigenic determinants preferentially expressed on normal and neoplastic cells of the melanocyte lineage and seen in association with HLA-A2.

Discussion

The results of this study indicate that some HLA class I–restricted CTL clones from melanoma patients can recognize HLA-A2$^+$ normal and neoplastic cells of the melanocyte lineage. This suggests that at least some of the antigens detected by T lymphocytes from melanoma patients might be related to the expression of tissue-related genes. A possibility is that peptides seen by the CTL clones in association with HLA-A2 derive from the processing of proteins encoded by genes preferentially expressed in cells of the melanocyte lineage. Alternatively, one should hypothesize the existence of tissue-specific differences in the mechanisms of antigen processing. These differences might lead, in different cell types, to the production and assembly with HLA-A2 of different peptides not necessarily originating from tissue-specific proteins. Both these hypotheses are in agreement with the lack of significant reactivity observed by CTL clones as A75, A83, and A94 on most HLA-A2$^+$ normal and neoplastic cells of nonmelanocyte antigen.

The experiments performed with quiescent melanocytes

suggested that the HLA-A2–restricted CTL clones recognize antigens whose expression is not restricted to proliferating cells. This is true for the significant lysis seen up to 144 h after seeding melanocytes in conditions that completely arrest their proliferation. We cannot rule out the possibility that CTL-mediated recognition of the quiescent melanocytes may disappear after 144 h of culture in conditions of growth arrest. However, experiments of CTL-mediated lysis on quiescent cells beyond the 144 h limit could not be performed because of the death of the melanocytes when cultured in the basal medium for periods longer than 6 d. On the other hand, our data indicate that the same CTL clones that can recognize the HLA-A2$^+$ melanocytes can also kill the fresh, uncultured autologous melanoma. This is consistent with the recognition of antigens expressed in vivo at least by the neoplastic cells. Clearly, it remains to be seen whether fresh, uncultured melanocytes, directly purified from patients' tissues, can be recognized by these CTL clones. In fact, the availability of autologous, uncultured melanocytes would allow one to perform the critical experiment that may formally prove whether antigens recognized by T cells on melanoma are also expressed in vivo on normal cells of the same tissue. A finding of CTL lysis on fresh uncultured melanocytes might strengthen the hypothesis that an immune mechanism could lead to destruction of melanocytes and melanoma in vivo and might also be interpreted as an indication that it is possible to alter an existing state of tolerance to tissue-related self antigens. Indeed, these hypotheses are in agreement with the development of an unexpected form of toxicity recently described in a proportion of melanoma patients subjected to a protocol that sequentially combined chemotherapy with IL-2 plus IFN-α (28). In fact, those authors reported for the first time that a depigmentation, defined as "vitiligo-like" and surely resulting from melanocyte destruction, occurred in a proportion of the treated patients. More importantly, this "toxicity" correlated significantly with tumor regression. These results suggested to the authors (28) that the combination of chemotherapy with immunotherapy, but not each of the two components alone, might activate a process leading to in vivo destruction of normal and neoplastic cells of the melanocyte lineage. Thus, although the precise mechanism of melanoma and melanocyte destruction in vivo remains to be elucidated, the possibility of an immune recognition of antigens shared between normal and neoplastic cells of the melanocyte lineage is supported by our results.

The findings described here add new information on the antigenicity of human melanoma and on the role of HLA-A2 molecule in the immune recognition of this tumor. In fact, the available data suggest that the HLA-A2 molecule is an immunodominant class I allele for presentation of melanoma antigens to autologous cytotoxic T cells (29), and that common melanoma antigens can be expressed by HLA-A2$^-$ melanomas after transfection with HLA-A2 (30). These data indicated that most human melanomas are antigenic and that most tumors from HLA-A2$^+$ patients may share common melanoma antigens seen by cytotoxic T cells. Our results confirmed the data on the existence of common melanoma antigens seen in association with HLA-A2. However, the findings described here suggest also that at least a proportion of these common antigens detected by patients' T cells on the tumor are not necessarily expressed as consequence of neoplastic transformation, but may represent a property of the tissue of origin of the tumor, since cultured normal melanocytes can be recognized by the same CTL clones that recognize all HLA-A2$^+$ melanomas. A possibility is that the origin of the antigens recognized by our CTL clones on melanoma and melanocytes may be linked to the differentiation program of cells in the melanocyte lineage. In fact, antigens expressed on newborn and fetal, but not adult melanocytes, were found also on the subset of tumors characterized as being in an intermediate stage of differentiation (18). With respect to this classification, it is interesting that we used neonatal foreskin melanocytes as targets for the screening of CTL clones. If antigens detected by T cells on melanoma are associated to the differentiation program of melanocytes, as it happens for serologically defined antigens, then it is possible that the CTL clones described in this study are directed to antigens that may be differentially expressed among normal or neoplastic melanocytes corresponding to distinct phases in the differentiation program of this lineage. This hypothesis implies that the heterogeneity for differentiation stage, commonly found among melanomas from different patients (18), should correlate with heterogeneity for expression of T cell–defined antigens. Therefore not all HLA-A2$^+$ melanomas may be recognized by CTL clones such as those described in our study. This is in agreement with the observation that one out of nine HLA-A2$^+$ melanomas was not lysed by the HLA-A2–restricted CTL clones. Similar possibilities exist for the recognition of melanocytes. However, the issue of differential expression of tissue related antigens on normal melanocytes might be settled only by testing the CTL clones on HLA-A2$^+$ melanocytes isolated from fetal, newborn, and adult skin.

In addition, the lack of lysis seen on one (Me4855) of the HLA-A2$^+$ melanomas might not be dependent on antigenic heterogeneity linked to the differentiation stage of the tumors. In fact, some patients may express rare HLA-A2 alleles that cannot be discriminated serologically, but that are readily distinguished by T cells (31), since the HLA-A2 subtype polymorphism produces changes in the peptide-binding groove that can affect peptide presentation (31). Therefore, it is possible that absence of recognition of some melanomas by the CTL clones described in this study is dependent on the presence of distinct HLA-A2 subtypes in the population. The answer to this possible explanation of heterogeneity in CTL-mediated recognition of melanomas will be obtained by applying a recently described technique of DNA typing for HLA-A2 subsets (32).

We do not know whether all HLA-A2–restricted CTL clones from each patient will kill HLA-A2$^+$ melanocytes. In fact, it is possible that the CTL clones detected in this study may represent only a fraction of the HLA-A2–restricted killers and that other HLA-A2–restricted CTL clones may kill the tumor but not HLA-matched melanocytes. Only the screening

Table 4. *Specificity of CTL Clones A75, A83, A94, and 119*

Targets				CTL clones				
Designation	Histology	HLA-A2 expression	mAb to:	A75	A83	A94	119	LAK
Me9742	Metastatic melanoma	96*	–‡	43§	61	41	33	50
Me9742			CD3**	14‖	25‖	14‖	0‖	62
Me9742			CD3***	15‖	25‖	13‖	1‖	47
Me9742			HLA-I	4‖	7‖	2‖	0‖	58
Me9742			HLA-DR	41	56	41	36	62
Fresh Me9742	Metastatic melanoma	96	–	13	27	16	13	26
Me13924	Metastatic melanoma	95		31	43	28	0	44
Me18732	Metastatic melanoma	95		33	43	43	3	53
Me8959	Metastatic melanoma	95		34	39	32	0	30
Me16938	Metastatic melanoma	93		59	68	61	3	55
Me4855	Metastatic melanoma	88		4	6	7	3	37
Me10538	Primary melanoma	95		26	45	30	9	66
Me3046/2	Metastatic melanoma	92		65	67	63	1	47
Me14932	Metastatic melanoma	94		27	54	26	1	62
Me5810	Primary melanoma	98		23	35	23	0	51
MeLatt	Metastatic melanoma	0		2	3	2	0	19
Me1402/R	Primary melanoma	0		0	0	0	0	38
Me4024	Metastatic melanoma	0		0	2	0	2	49
Me665/1	Metastatic melanoma	0		0	10	0	3	66
Me9460	Metastatic melanoma	0		0	5	0	4	53
Me13443	Metastatic melanoma	0		0	2	0	0	53
Me1340	Metastatic melanoma	0		0	0	0	1	43
Me1811	Metastatic melanoma	4		1	4	0	2	58
Me4405	Primary melanoma	0		1	9	1	5	66
Reca458	Renal carcinoma	82		0	8	0	4	67
RecaSor	Renal carcinoma	93		1	2	0	2	69
RecaMar	Renal carcinoma	17		0	4	0	3	60
HT29	Colon carcinoma	0		0	0	1	0	62
A431	Epidermoid carcinoma	0		0	5	0	3	57
SKBR3	Breast carcinoma	13		0	5	0	5	37
CALU3	Lung carcinoma	91		0	2	0	0	57
Ovca432	Ovarian carcinoma	2		0	0	0	0	61
SKOV3	Ovarian carcinoma	91		0	1	0	0	46
IGROV1	Ovarian carcinoma	0		0	5	0	3	57
N592	Small cell lung carcinoma	10		0	2	2	2	68
H446	Small cell lung carcinoma	0		0	3	0	0	28
Re458	Normal kidney cells	83		0	7	0	3	67
F1338/1	Fibroblast	86		0	9	0	9	48
F8536	Fibroblast	0		1	1	1	0	35
F9203	Fibroblast	0		0	2	0	1	45
F3046	Fibroblast	53		0	14	0	8	68

continued

Table 4. *(continued)*

Targets				CTL clones				
Designation	Histology	HLA-A2 expression	mAb to:	A75	A83	A94	119	LAK
LCL9742	EBV-transformed B cell	95		0	2	0	0	60
LCL10538	EBV-transformed B cell	85		0	0	0	0	46
LCL1811	EBV-transformed B cell	2		0	0	0	0	37
LCL4405	EBV-transformed B cell	0		0	3	0	0	21

Specificity of CTL clones A75, A83, A94, and 119 from patient 9742 was tested in a 4-h ^{51}Cr-release assay. LAK cells were produced as described in the legend to Table 2.

* Reactivity of different cells with the mAb CR11.351 (anti-HLA-A2) is expressed as percent positive cells by cytofluorimetric analysis. Positive reaction with CR11.351 was confirmed also by the mAb anti-HLA-A2 PA2.1, BB7.2, and MA2.1. All targets in the panel are positive for HLA class I antigens as detected by the mAb W6/32 (10).

‡ Inhibition of Me9742 lysis was performed by preincubating the effectors with 1 μg/ml of anti-CD3 mAb OKT3(**) or TR66(***), and preincubating the target with 1 μg/ml of W6/32 (anti-HLA-class I), or L243 (anti-HLA-DR).

§ Results are expressed as percent lysis. Underlined values represent significant values of lysis (SNK test, $p = 0.01$).

‖ Lysis in the presence of antibodies is significantly different from lysis of the same target in the absence of antibody (SNK test, $p = 0.01$). LCL9742, LCL10538, LCL1811, and LCL4405 are lymphoblastoid cell lines autologous respectively to the tumors Me9742, Me10538, Me1811, and Me4405. Re458 kidney cells and fibroblasts F3046 are autologous respectively to the tumors Reca458 and Me3046/2.

of a large panel of HLA-A2–restricted CTL clones for lysis of melanoma and melanocytes will indicate whether the HLA-A2 molecule is a restriction element only for antigens shared between melanoma and melanocytes, or also for antigens expressed on the neoplastic cells only.

The CTL clones with reactivity on normal melanocytes were found not only in PBL but also in TIL. This indicates that HLA-A2–restricted T cells with the ability to recognize normal and neoplastic cells of the melanocyte lineage are not confined to a single tissue but can be found either in the peripheral circulation or the tumor site. However, the analysis performed by T cell clones, due to the strong selection that occurs during the cloning procedure, does not allow us to draw any conclusion on the issue of frequency of these effectors either in the PBL or in the TIL populations. This issue may be addressed only by analysis of the molecular structure of the TCR expressed by these effectors (Sensi, M., et al., manuscript in preparation).

Finally, the results of the specificity assay performed with CTL clone 119 indicated that HLA-A2 is not the only restricting element for recognition of TAA on tumor 9742 and that some of the T cell–defined antigens of this tumor may not be frequently shared with allogeneic cells even within the melanocyte lineage. This suggests that the antigenicity of a human melanoma, as defined by autologous cytotoxic T cells, may be a complex phenotype resulting not only from heterogeneity in the tissue distribution and molecular origin of the antigens, but also from the expression on the same tumor of common or unique antigens, and from the presence of distinct peptide–HLA complexes.

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Address correspondence to Dr. Andrea Anichini, Division of Experimental Oncology D, Istituto Nazionale Tumori, Via Venezian 1, 20133 Milan, Italy.

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