

## LYSIS OF HUMAN MELANOMA CELLS BY AUTOLOGOUS CYTOLYTIC T CELL CLONES

Identification of Human Histocompatibility Leukocyte Antigen A2 as a  
Restriction Element for Three Different Antigens

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Tumor-specific transplantation antigens (TSTA)<sup>1</sup> as target structures for a potent autologous immune rejection response have been regularly found in chemically and UV-induced mouse tumors. These antigens were defined by in vivo crossprotection experiments (1, 2). There is experimental evidence suggesting that TSTA are recognized by cytolytic T lymphocytes (CTL) in the course of immunologically mediated tumor regression (3, 4). Serological probes against TSTA could not be obtained. Recently, the isolation of methylcholanthrene-induced TSTA was reported (5). In the search for similar antigens on human melanoma cells and on other human cancers, several groups derived tumor-reactive CTL clones from the peripheral blood of tumor patients (6–8) (for review see reference 9). Improved culture techniques, meanwhile, facilitate a more systematic production of stable tumor-reactive CTL clones (10). To date, nothing is known about the nature of their target antigens.

Two principal target structures for CTL on autologous target cells are characterized at a molecular level. First, fragments of viral antigens or mutated self proteins are recognized in association with MHC molecules (11–13) and, second, mutated MHC molecules themselves can be the target molecules (14). So far, the mechanism of CTL recognition of autologous human tumor cells has been investigated by inhibition experiments with mAbs against common TCR structures and framework determinants of HLA molecules. For a subset of cytotoxic lymphocyte clones, TCRs and HLA class I molecules were clearly involved in target cell recognition (for review see reference 9).

In continuation of earlier work (7), we recently generated CTL clones from a melanoma patient (AV) by mixed lymphocyte tumor cell culture (MLTC) and subse-

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<sup>1</sup> *Abbreviations used in this paper:* CTL, cytolytic T lymphocytes; MLTC, mixed lymphocyte tumor cell culture; TSTA, tumor-specific transplantation antigens.

quent cloning of responder lymphocytes using autologous tumor cells as stimulators (15). The CTL clones lysed the autologous melanoma cells but not autologous EBV-transformed B cells, and none of the allogeneic targets within a panel of ~20 tumor lines of various histologic origins. No difference in tumor antigen expression was detected on autologous melanoma clones. To find out if the CTL clones were directed against different antigens, immunoselection experiments were carried out with two of the CTL clones (CTL2/9 and CTL5/57). Some of the surviving melanoma clones were resistant to lysis by the CTL clones used for immunoselection, but were lysed by other autologous CTL clones. Thus, we concluded that at least three stable CTL-defined antigens are simultaneously expressed on SK-MEL-29 cells. These findings parallel those obtained with the methylcholanthrene-induced mouse mastocytoma P815 where multiple simultaneously expressed tumor antigens were also detected in immunoselection experiments (3).

In the human melanoma line SK-MEL-29, antigen A is recognized by CTL2/9 and antigen B by CTL5/57. Antigen C is recognized by the other CTL clones included in our recent study (CTL3/7, CTL3/9, and CTL5/76) and may be dissected into further specificities by immunoselection experiments. The lysis by all CTL clones could be inhibited by the mAb W6/32 (16), which is directed against a monomorphic determinant of HLA class I molecules. This suggests that HLA class I molecules are involved in antigen recognition by the AV CTL. Here we demonstrate experimental evidence that all previously defined AV melanoma antigens are presented to autologous CTL in association with HLA-A2.

## Materials and Methods

**Patient.** Patient AV, a 36-yr-old caucasian male, developed metastatic malignant melanoma in 1975. He underwent resections of extensive metastatic melanoma of axillary, supraclavicular, and cervical lymph nodes in 1975 and in 1978. Since that time he is free of detectable disease. In 1975 a melanoma cell line (SK-MEL-29) was derived from a metastatic lesion. From 1976 to 1978, the patient's lymphocytes were found to be strongly cytotoxic for autologous cultured melanoma cells (17). Later, only minimal lymphocyte cytotoxicity for autologous melanoma cells was detectable. The patient's HLA-A,-B,-C phenotype is A2, A28, B44 (Bw4), Bw50 (Bw6), Cw5, Cw6.

**Cell Lines.** The melanoma cell line SK-MEL-29 (17) was a generous gift of Dr. L. J. Old (Memorial Sloan-Kettering Cancer Center, New York). The derivation of melanoma clones from this line by limiting dilution and in vitro immunoselection is explained in Fig. 1. The human melanoma line MZ-2-MEL was described in reference 10. AK-EBV and AV-EBV are EBV-transformed lymphoblastoid (EBV-B) cell lines established from a healthy donor (AK) and from the melanoma patient (AV), respectively. Tumor cell lines were maintained in DME (Gibco Laboratories, Grand Island, NY) containing 10 mM Hepes buffer, L-arginine (116 mg/ml), L-asparagine (36 mg/ml), L-glutamine (216 mg/ml), penicillin (10 IU/ml), streptomycin (100  $\mu$ g/ml), and 10% FCS. This medium is further referred to as M<sup>a</sup>. EBV-B lines were cultured in RPMI 1640 (M<sup>b</sup>), supplemented as described for M<sup>a</sup>. Cell cultures were kept in a water-saturated atmosphere with 5% CO<sub>2</sub> at 37°C.

When indicated, human rIFN- $\gamma$  (Genentech, San Francisco, CA) was added to the culture medium at a concentration of 100 U/ml 4 d before cytolysis assays to increase the level of HLA expression on target cells.

**Maintenance of CTL Clones.** The derivation of autologous CTL clones directed against SK-MEL-29 was described previously (15). CTL were maintained in long-term culture by transferring (every 4–7 d)  $2-3 \times 10^5$  CTL to 2-ml cultures containing  $5 \times 10^4$  autologous melanoma cells as stimulators and  $2 \times 10^5$  AK EBV cells as feeders in 24-well tissue culture plates. Both stimulator and feeder cells were irradiated before CTL culture with 100 Gy from a

$^{137}\text{Cs}$  source. As culture medium ( $\text{M}^c$ ), we used RPMI 1640 medium supplemented as described above, but with 10% human serum and human rIL-2 (25 U/ml) (a generous gift from Biogen S. A., Geneva, Switzerland) (18).

**Derivation of CTL Clones.** MLTC experiments and cloning of responder lymphocytes and testing for cytolytic activity were performed as described previously (10, 15). PBL were separated from heparinized blood of the patient AV by centrifugation on a Ficoll-Paque gradient. PBL ( $10^6$ ) were cocultured with  $10^5$  melanoma cells (clone SK-MEL-29.1.29, irradiated with 100 Gy from a  $^{137}\text{Cs}$  source) in 2 ml of medium  $\text{M}^c$  containing 10% human serum on 24-well plates (Greiner, Nürtingen, FRG). Human rIL-2 was added from day 3 on at 25 U/ml.

After 7 d, MLTC responder lymphocytes were seeded at 1 cell/well in round-bottomed 96-well microtiter plates (Greiner) preseeded with autologous melanoma cells ( $3 \times 10^3$ /well, irradiated) as stimulators and AK EBV ( $10^4$ /well, irradiated) as feeder cells in a total volume of 200  $\mu\text{l}$  of medium  $\text{M}^c$  with human serum and IL-2. 1 wk later (day 7), 100  $\mu\text{l}$  of supernatant was removed from each well and replaced by 100  $\mu\text{l}$   $\text{M}^c$  (with IL-2) with fresh stimulator and feeder cells. On day 14, microcultures were transferred to flat-bottomed 96-well microtiter plates (Greiner) (total culture volume, 300  $\mu\text{l}$ ) and tested on day 21 in an initial screening for cytolytic activity on autologous melanoma cells and K562 as described below. Lymphocyte colony growth was followed under microscope from day 12 on. Exceptionally fast-growing colonies were transferred to Linbro wells immediately.

**Assay for Cytolytic Activity.** Standard  $^{51}\text{Cr}$  release cytotoxicity assays were used (19). Target cells were incubated at  $10^7$  cells/ml in FCS for 45 min at  $37^\circ\text{C}$  with 200  $\mu\text{Ci}/\text{ml}$  of  $\text{Na}^{(51}\text{Cr})\text{O}_4$  (Institut des Radioelements, Fleurus, Belgium), washed three times, and resuspended in medium  $\text{M}^a$  at  $10^4$  cells/ml. CTL were serially diluted in duplicates in 100  $\mu\text{l}$  of medium  $\text{M}^a$  in conical 96-well microtiter plates. After adding 100  $\mu\text{l}$  of labeled target cell suspension, plates were incubated for 4 h at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  atmosphere. The assays were terminated by centrifugation at 200  $g$  for 5 min and collection of 100  $\mu\text{l}$  supernatant for counting in a gamma counter. Percent specific  $^{51}\text{Cr}$  release was calculated with the formula: percent specific  $^{51}\text{Cr}$  release =  $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$ . Spontaneous  $^{51}\text{Cr}$  releases were between 10 and 25% of the total label incorporated in the cells.

MLTC-derived responder colonies were initially screened in a microcytotoxicity assay. Carefully mixed cell suspensions in microtiter plates were split in 60- $\mu\text{l}$  aliquots and added to labeled targets in microtiter test plates to a final volume of 200  $\mu\text{l}$ . The plates were processed as described above.

**Immunoselection with CTL Clones.** Immunoselection experiments were designed according to Maryanski and Boon (20) and were performed in our previous study (15).

**Serological Reagents.** Human HLA-reactive pregnancy sera were identified according to standard procedures (21). The mAbs used in this study are listed in Table I. They were purchased from the American Type Culture Collection (Rockville, MD), except for the mAb 4E, which is a kind gift of Dr. S. Y. Yang (Memorial Sloan-Kettering Cancer Center). All mAbs were used as ascites with an approximate protein concentration of 15–20 mg protein per ml.

**Inhibition of Cytolysis.**  $^{51}\text{Cr}$ -labeled target cells ( $10^3$ /well) were admixed in duplicates to serial dilutions of antisera or mAbs in medium  $\text{M}^a$  in conical 96-well microtiter plates. Then, CTL ( $3 \times 10^4$ /well) were added in the same medium to give a final volume of 200  $\mu\text{l}$ /well. After a 4-h incubation period at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  atmosphere, plates were harvested and processed as described for cytotoxicity assays. Controls always included incubation of antisera or mAbs at the maximum concentrations with targets alone to exclude antibody-dependent mechanisms of cell lysis in the absence of CTL.

**Flow Cytometric Analysis.** Tumor cells were incubated with murine mAbs at saturating concentrations for 30 min on ice, washed, and stained with polyclonal goat anti-mouse F(ab')<sub>2</sub> antibody fragments coupled with FITC (Coulter Electronics Inc., Hialeah, FL) for another 30 min on ice. Samples were analyzed on an EPICS C cell sorter (Coulter Electronics Inc.).

**DNA Extraction and Southern Blot Hybridization.** High-molecular weight DNA was prepared from tumor cells and CTL as described elsewhere (26). CTL were separated from cell debris on a Ficoll-Paque gradient (Pharmacia Fine Chemicals, Uppsala, Sweden) before DNA extraction. Hybridization with bacteriophage M13 was performed as detailed in reference 27.

**Immunoprecipitation and IEF Analysis.** Cells were metabolically labeled with [<sup>35</sup>S]methionine. Preparation of NP-40 extracts and immunoprecipitation with W6/32 (16) and formalin-fixed *Staphylococcus aureus* Cowan strain 1 was described earlier (28). IEF of the immunoprecipitates was carried out in polyacrylamide gels according to the 10th International Histocompatibility Workshop protocol (29). HLA antigens of the cell lines were assigned by IEF band patterns in comparison with the standardized IEF band patterns of HLA class I alleles of the 10th International Histocompatibility Workshop (29).

## Results

**Inhibition of Cytolysis by Human Anti-HLA-A2 Sera.** In mouse model systems inhibition of CTL lysis by anti-MHC antibodies proved to be a reliable approach in identifying MHC molecules as restriction elements (30–32). The application of this approach to human systems is hampered by a lack of allele-specific anti-HLA mAbs. Therefore, we primarily used HLA-reactive sera from pregnant women. About 10% of pregnancy sera exhibit mono- or multispecific HLA reactivity. Among these, anti-HLA-A2 sera are found most frequently and are predominant among monospecific sera (33). Since AV cells express HLA-A2, we tested monospecific anti-HLA-A2 sera from pregnant women in inhibition experiments with the effector CTL2/9 and the target melanoma clone SK-MEL-29.1. At a 16% serum concentration, 4 of 10 anti-HLA-A2 sera reduced the CTL lysis by >60% in 4-h <sup>51</sup>Cr release assays at a 30:1 E/T ratio (see example in Fig. 2).

Absorption of an HLA-A2-reactive serum with an excess of mouse mastocytoma P1.HTR cells (34) that expressed an HLA-A2 gene after transfection (35, 36) abolished its inhibiting effect (P1.HTR-A2 cells were a kind gift from J. Maryanski, Ludwig

TABLE I  
*mAbs Used in Inhibition Experiments and FACS Analysis*

mAb	Antigenic specificity	Isotype	Reference
W6/32	Class I HLA	IgG2a	16
HB55	Class II HLA	IgG1	22
MA2.1	HLA-A2, HLA-B17	IgG1	23
PA2.1	HLA-A2, HLA-A28	IgG1	24
4E	HLA-B	IgG2a	25

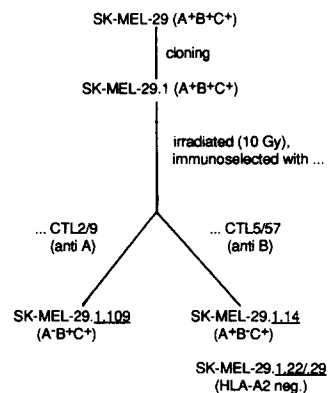


FIGURE 1. AV melanoma clones derived by limiting dilution and immunoselection with autologous CTL clones (15).

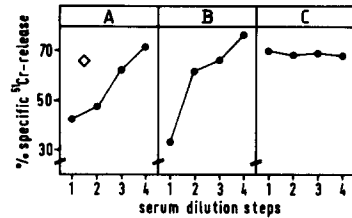


FIGURE 2. Inhibition of CTL lysis by human anti-HLA-A2 pregnancy serum. Serum (HbF-3423) was added at a starting dilution of 1:2 to the cytotoxicity assay (effector, CTL2/9; target, SK-MEL-29.1; E/T, 30:1 for 4-h incubation; ◇, no serum added (A). Serum B was absorbed to P1.HTR cells (untransfected) and serum C to P1.HTR-A2 (expressing HLA-A2 after transfection) before the cytotoxicity test. Absorption was performed by mixing  $10^9$  cells with 0.9 ml of serum and incubating the suspension for 1 h at room temperature vigorously shaken at regular intervals. Dots represent means of triplicates.

Institute for Cancer Research, Lausanne, Switzerland). Absorption with untransfected P815 cells did not influence the inhibition (Fig. 2). This indicated that the blocking ability of the serum was due to its HLA-A2 reactivity.

**Inhibition of Cytotoxicity by Anti-HLA-A2 mAbs.** A number of HLA-A2-reactive mAbs have been isolated. Two of them, MA2.1 (23) and PA2.1 (24), were used as ascites for inhibition experiments in this study (Table I). MA2.1 recognizes an antigenic determinant shared by HLA-A2 and -B17 (23). PA2.1 is directed against a specific determinant shared by HLA-A2 and a variant of HLA-A28 (37) that is different from the epitope recognized by MA2.1 (38). MA2.1 could efficiently inhibit the lysis by all five previously established AV CTL clones (15) (Fig. 3 for CTL2/9, 5/57, and 5/76). PA2.1 only reproducibly inhibited the lysis by CTL clone CTL2/9. This indicated the involvement of HLA-A2 in target recognition by all of the CTL clones.

Tumor cell variants SK-MEL-29.1.109 and SK-MEL-29.1.14 were derived by immunoselection experiments with CTL2/9 and CTL5/57 (Fig. 1). These variants were resistant to lysis by the selecting CTL but still lysed by others (15; and Fig. 4 A). As demonstrated by FACS analysis with the anti-HLA-A2 antibodies MA2.1 and PA2.1, these antigen-negative variants still expressed HLA-A2 (Fig. 5).

**Lack of HLA-A2 Expression on AV Melanoma Cells Abrogates Cytotoxicity by Autologous CTL Clones of Different Specificities.** In vitro immunoselection experiments were previously performed with CTL2/9 and CTL5/57 (15; and Fig. 1). Cells of the AV melanoma clone SK-MEL-29.1 were irradiated with 1,000 rad to increase the probability of genetic lesions causing a loss of expression of CTL-defined antigens. These target cells were incubated with a 10-fold excess of CTL (15). After immunoselection with CTL 5/57, 13 survivor clones were found out of  $10^5$  initial cells plated at  $10^3$  cells/well (series A), and 17 surviving tumor cell clones were found out of  $2 \times 10^5$  initial cells plated at  $2 \times 10^3$  cells/well (series B). Two of the survivors, SK-MEL-29.1.22 (out of series A) and -29.1.29 (out of series B) were resistant to the selecting CTL5/57, as well as to all other CTL clones established so far. Lysability of these

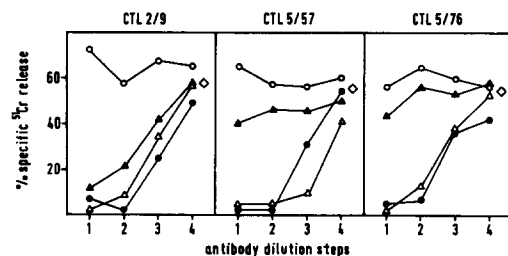


FIGURE 3. Inhibition of CTL lysis by anti-HLA-A2 mAbs. Cytotoxic activity of CTL clones directed against three different tumor antigens was tested in the presence of various mAbs (target, SK-MEL-29.1; E/T, 30:1; for 4-h incubation). Antibodies were added in ascites form at a starting dilution of 1:100. (●) W6/32; (Δ) MA2.1; (▲) PA2.1; (○) negative control; (◇) no antibody; for further information about mAb see Table I).

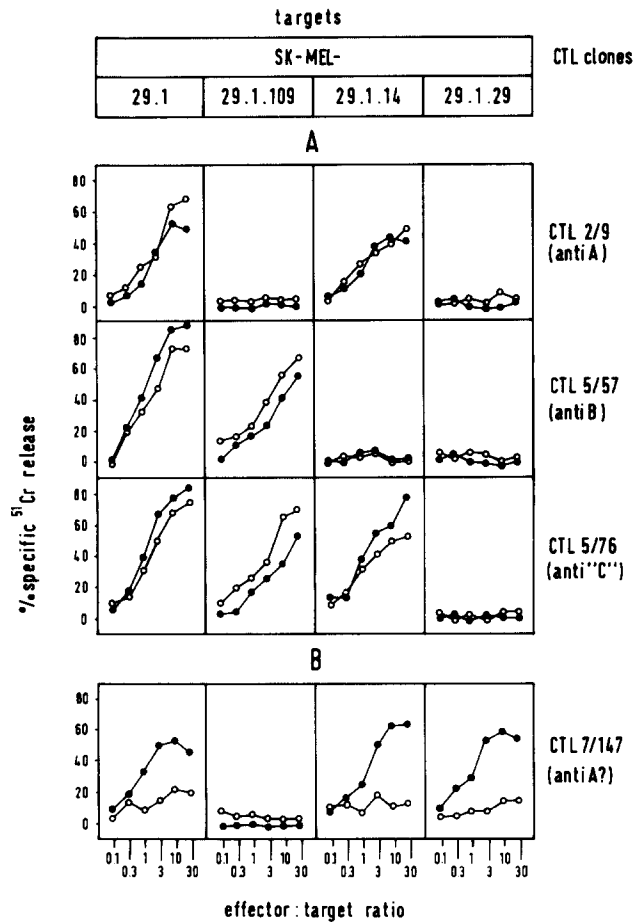


FIGURE 4. Cytolytic activity of AV CTL clones against immunoselected tumor cell variants. For information about target melanoma clones see Fig. 1. CTL in A were derived from MLTC experiments with SK-MEL-29.1 as stimulator. CTL7/147 (B) was derived in the course of a MLTC experiment with SK-MEL-29.1.29. Targets were pretreated with IFN- $\gamma$  (●) (100 U/ml for 4 d) or untreated (○).

tumor cell variants could not be restored by pretreatment with IFN- $\gamma$  (Fig. 4 A for three of five CTL clones). Since these two tumor cell variants behaved identically in all CTL lysis tests and FACS analyses described below, only the data obtained with SK-MEL-29.1.29 are shown. SK-MEL-29.1.22 and -29.1.29 additionally lost the growth-stimulating ability for the CTL clones (not shown). We performed a DNA fingerprint analysis to demonstrate that the tumor cell variants resistant to lysis by different autologous CTL specificities are of the same genetic origin as the parental melanoma cell clone. The bacteriophage M13, generally used as a cloning vector for DNA sequencing, was reported to hybridize to a distinct set of hypervariable minisatellites in the genome of a variety of species. It is used as a probe to detect individual specific hybridization patterns in Southern blot analysis (39). As shown in Fig. 6, the patterns obtained with DNA from SK-MEL-29.1.22 and -29.1.29 correspond to the parental melanoma cell clone SK-MEL-29.1, to AV CTL clones, and to the AV EBV-B cells, indicating identical genetic origin. The hybridization patterns of allogeneic cell lines could be clearly distinguished from AV cells.

According to the results described above, HLA-A2 is directly involved in the recognition of three different AV melanoma antigens. Loss of HLA-A2 expression should

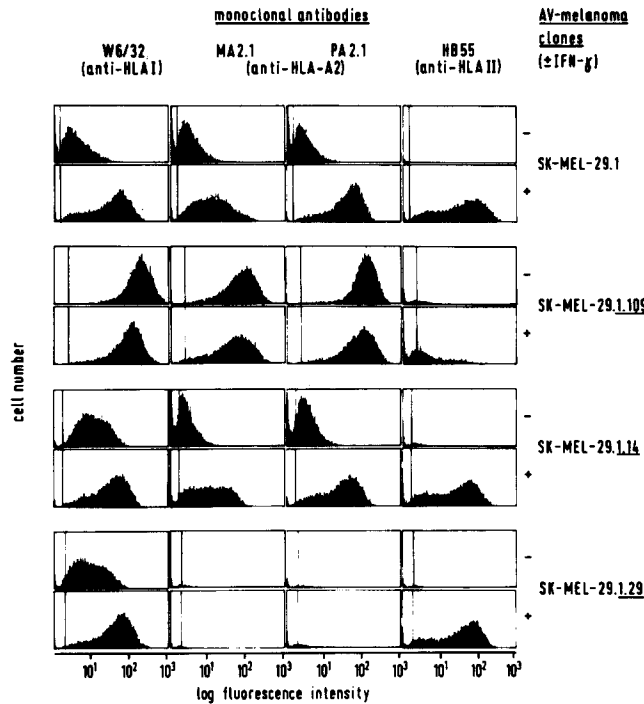


FIGURE 5. FACS analysis of immunoselected AV melanoma clones. For information about the derivation of tumor cell clones and about antibodies see Fig. 1 and Table I, respectively. Where indicated, tumor cells were pretreated with IFN- $\gamma$  at 100 U/ml for 4 d. 5,000 stained cells per sample were analyzed. The fluorescence intensity is displayed on a logarithmic scale in channels.

then result in resistance to lysis by all CTL specificities. A FACS analysis was performed on the immunoselected variants (Fig. 5). All the melanoma clones expressed HLA class I molecules. However, the variant clones SK-MEL-29.1.22 (not shown) and 1.29 did not express HLA-A2 at a detectable level as tested with two anti-HLA-A2 mAbs (MA2.1 and PA2.1) binding to different epitopes on HLA-A2 (38). Pretreatment with IFN- $\gamma$  induced the expression of HLA class II molecules and increased the overall expression of HLA class I molecules on all tumor cell clones, but did

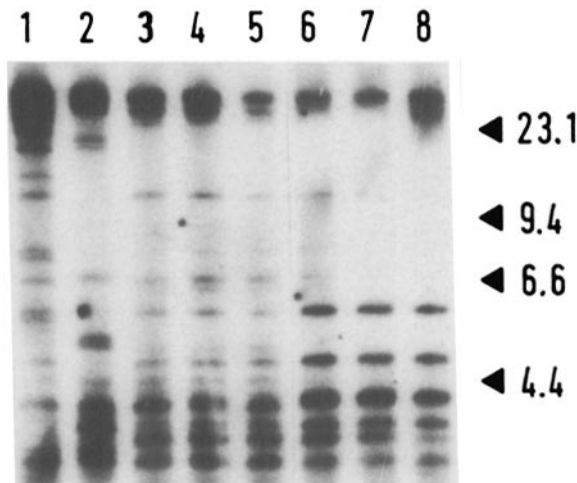


FIGURE 6. Fingerprint analysis with bacteriophage M13 DNA. DNA of various cell lines was digested with Hinf I (59), separated on a 0.7% agarose gel, transferred to nitrocellulose filters, and hybridized with <sup>32</sup>P-labeled M13 DNA according to reference 28. Lane 1, MZ-2-MEL; lane 2, AK-EBV; lane 3, AV-EBV; lane 4, CTL2/9; lane 5, CTL5/76; lane 6, SK-MEL-29.1; lane 7 and 8, SK-MEL-29.1.22 and 1.29. Hind III fragments of  $\lambda$  phage DNA were used as size markers (fragment lengths in kilobases).

not induce HLA-A2 expression on clones 1.22 (not shown) and 1.29. In addition, IEF analysis of HLA class I antigens immunoprecipitated with W6/32 from the melanoma clone SK-MEL-29.1.29 confirmed the loss of HLA-A2 expression. This melanoma clone also lacked expression of HLA-B44 without IFN treatment (Fig. 7). Cytofluorographic characteristics and the resistance to lysis by the HLA-A2-restricted AV-CTL clones were repeatedly controlled and found to be stable over a continued culture period of 1 yr.

*Derivation of AV CTL Clones that Involve an HLA-B Molecule in Lysis of Autologous Tumor Cells; Impact of IFN- $\gamma$  on their Lytic Activity.* To find out whether additional HLA molecules are involved in autologous target recognition, MLTC experiments were performed with AV PBL and the autologous variant melanoma clone SK-MEL-29.1.29, which did not express HLA-A2. Responder lymphocytes were cloned after 7 d. Of 1,000 wells seeded at one responder cell per well, 156 proliferating clones were obtained. Among 15 responder clones with lytic activity, eight clones lysed SK-MEL-29.1.29 but not K562 and not autologous EBV-transformed B cells. Two fast growing CTL clones, CTL7/4 and CTL7/147, were chosen for further studies.

In repeated tests both CTL clones lysed SK-MEL-29.1.29 cells only weakly. The specific  $^{51}\text{Cr}$  releases did not exceed 25% at an E/T ratio of 30:1. However, pretreatment of tumor cells with IFN- $\gamma$  restored the lysis in a dose-dependent manner. Optimal lysis was seen at an IFN concentration of 100 U/ml for 4 d in tumor cell culture medium. K562 and AV EBV cells remained resistant to lysis after exposure to IFN. The HLA-A2 $^{+}$  AV melanoma clones SK-MEL-29.1 and -29.1.14 were only effectively lysed after exposure to IFN- $\gamma$ . This indicated that the low level of expression of the target structure(s) recognized by the newly established CTL clones is a common trait of SK-MEL-29 cells. Fig. 4 B summarizes the lysis of AV melanoma clones. Only the results obtained with CTL7/147 are shown since both CTL behaved identically in all tests.

The tumor cell variant SK-MEL-29.1.109, which is negative for the CTL-defined antigen A, was resistant to lysis by both CTL, even after pretreatment with IFN- $\gamma$ . One explanation is that antigen A is presented to autologous CTL both in associa-

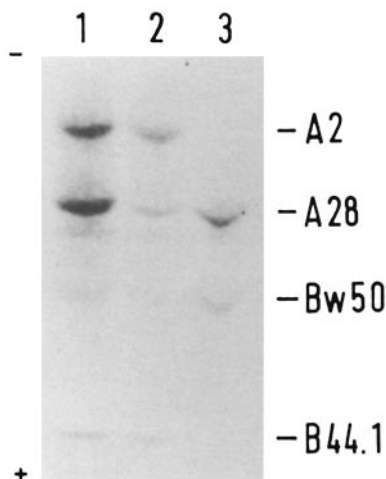


FIGURE 7. IEF analysis of immunoprecipitates from AV melanoma clones. Immunoprecipitation was performed with the mAb W6/32 (16). The fluorograph of the gel is oriented with the basic end at the top. Samples are lane 1, AV-EBV; lane 2, SK-MEL-29.1; lane 3, SK-MEL-29.1.29.



TABLE II  
*Involvement of Different Class I Molecules in the Recognition of SK-MEL-29 Cells  
 by Autologous CTL Clones as Indicated by Inhibition of Cytolysis*

CTL clone	Without antibody	W6/32	HB55	MA2.1	PA2.1	4E	NS-1
CTL2/9	66	0	65	3	11	68	55
CTL5/57	84	11	85	15	55	89	85
CTL7/4	70	0	74	68	68	0	82
CTL7/147	52	0	46	49	54	0	53

Antibodies were used in ascites form at a 1:100 dilution in a 4-h  $^{51}\text{Cr}$  release assay (E/T, 30:1). As target we used SK-MEL-29.1 pretreated with IFN- $\gamma$  at 100 U/ml for 4 d. Data are percent specific  $^{51}\text{Cr}$  release. Antigenic specificities of antibodies are indicated in Table I.

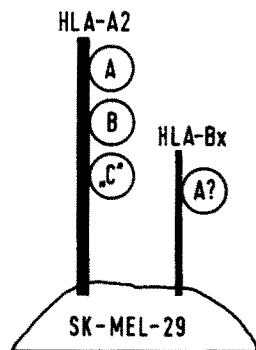


FIGURE 8. Diagram of CTL-defined antigens and HLA restriction elements on AV melanoma cells as deduced from antibody inhibition and immunoselection experiments.

tion with HLA-A2 and with an HLA-B molecule (Fig. 7). Alternatively, however less likely, the tumor cell clone SK-MEL-29.1.109 may have lost the expression of both antigen A and the respective HLA-B-molecule. Our present effort to identify the HLA-B-molecule involved in CTL recognition will perhaps answer this question. Lysis of IFN-treated SK-MEL-29.1 cells by CTL7/4 and CTL7/147 was inhibited by W6/32. Anti-HLA-A2 mAb had no effect. The mAb 4E (25), however, which is directed against a common HLA-B determinant, could inhibit CTL lysis, whereas this antibody had no effect on the lysis by HLA-A2-restricted CTL (Table II). The mAb 4E also blocked the lysis by CTL7/4 and 7/147 on IFN-treated SK-MEL-29.1.29 cells (not shown). The observation that MA2.1 could not inhibit lysis by these newly established CTL on an HLA-A2<sup>+</sup> target formally excluded a nonspecific inhibition of lysis by the previously established CTL.

### Discussion

In the human melanoma SK-MEL-29 (AV), at least three different antigens are recognized by autologous CTL in association with HLA-A2 (Fig. 8). Four HLA-A2<sup>+</sup> allogeneic melanoma lines included in a specificity analysis were found to be resistant to lysis by AV CTL (15). This indicates that the expression of these tumor antigens is not common in human melanoma. In a study on human sarcomas a common antigen was reported to be recognized by CTL in association with HLA-A2 (40).

HLA-restricted virus-specific CTL were shown to react with synthetic peptides of viral antigens bound to appropriate HLA molecules on uninfected target cells (12). This observation implies that CTL recognize viral antigens after processing to peptide fragments and association with MHC molecules. Point mutations of HLA-A2 were suggested to impair CTL recognition by interfering with peptide binding, altering the position of the respective peptide or changing the conformation of the peptide-HLA complex (41). In our previous experiments, AV melanoma variant clones were derived after mutagenesis and immunoselection that were only resistant to lysis by the selecting CTL clone. This was suggested to be due to loss of expression of certain tumor antigens (15). Regarding the findings reported here, however, it cannot be ruled out that mutations of the restricting HLA-A2 molecule that are undetectable by antibody binding might have occurred in "antigen loss" variants. Such mutations could affect tumor antigen binding and, thus, account for resistance of variant tumor cell clones to CTL lysis.

All AV CTL clones previously established in the course of three independent MLTC experiments with SK-MEL-29.1 stimulator cells are restricted by HLA-A2. AV CTL (CTL7/4 and 7/147) that do not depend on HLA-A2 for the recognition of autologous target cells were only derived by MLTC with the HLA-A2<sup>-</sup> melanoma clone 1.29. These CTL clones exhibit strong lytic activity when target cells are pretreated with IFN- $\gamma$ , which is known to induce HLA class I and II expression on human melanoma cells (42, 43). As indicated by antibody inhibition experiments, an HLA-B molecule is involved in target recognition by CTL7/4 and CTL7/147. On SK-MEL-29 cells the HLA-B locus is weakly expressed as compared with the HLA-A locus, and HLA-B expression can be strongly enhanced by IFN- $\gamma$  (unpublished results). Thus, the differential expression of HLA subregions can explain why HLA-A2 appears to be a dominant restriction element on SK-MEL-29 cells.

The importance of MHC restriction elements both for recognition by immune effectors *in vitro* and for progression of the tumors *in vivo* is well documented in several rodent tumor models (for review see reference 44). In human cancer, lacking or strongly reduced HLA class I expression was found in small cell lung carcinoma (45), neuroblastoma (46), mammary carcinoma (47, 48), skin carcinomas (49, 50), colorectal carcinoma (51), and B cell lymphoma (52). For the latter three a correlation between low class I expression and histologic criteria of malignancy was shown. Melanoma metastases frequently express HLA class I molecules at lower levels than the respective primary tumors (53, 54). Downmodulation of HLA class I expression in melanomas as well as in neuroblastomas seems to be correlated *in vitro* with high levels of *myc* oncogene expression, which can be reversed by IFN- $\gamma$  (55, 56). While all these observations concern pan-HLA class I expression on human tumors, there is so far little evidence for reduced or lacking expression of HLA subregions (57, 58). In the AV melanoma model we observed the loss of the product of a single HLA class I allele that presents multiple tumor antigens to autologous CTL. Such events may have an impact on tumor progression *in vivo*. The use of antibodies against locus- and allele-specific HLA determinants should help to evaluate in immunohistochemical studies whether loss of single HLA specificities occurs *in vivo* in metastatic disease.

### Summary

From the peripheral blood of the melanoma patient (AV), we derived cytolytic

T lymphocyte (CTL) clones that lysed the autologous tumor line SK-MEL-29, but not autologous EBV-B cells, K562, and other tumor targets. By immunoselection experiments it was shown that the CTL clones recognized at least three different antigens on the autologous tumor cells. We demonstrate here that these melanoma antigens are presented to the CTL in association with HLA-A2. First, HLA-A2-reactive pregnancy sera as well as an mAb against HLA-A2 inhibited the CTL lysis. Second, immunoselected melanoma subclones that were resistant to lysis by CTL clones against the three antigens described were found to lack expression of HLA-A2. By sensitizing the patient's lymphocytes against an HLA-A2<sup>-</sup> melanoma clone, we established a new series of CTL clones recognizing autologous AV melanoma cells. However, efficient lysis was only seen when target cells were pretreated with IFN- $\gamma$ . The lytic activity of these CTL was selectively inhibited by an mAb against a common HLA-B determinant. These results indicate that in addition to HLA-A2, other class I antigens are involved in the recognition of AV melanoma cells by autologous CTL.

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