The Tyrosinase Gene Codes for an Antigen Recognized by Autologous Cytolytic T Lymphocytes on HLA-A2 Melanomas

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Summary

Lymphocytes of melanoma patients can be restimulated in vitro with autologous tumor cells to generate antitumor cytolytic T lymphocytes (CTL). Previous reports have indicated that, when such CTL are obtained from HLA-A2 melanoma patients, they often display broad reactivity on A2 melanoma cell lines. Such antitumor CTL clones, which appeared to recognize the same antigen, were isolated from two patients. We report here the cloning of a cDNA that directs the expression of the antigen recognized by these CTL. This cDNA corresponds to the transcript of the tyrosinase gene. The gene was found to be active in all tested melanoma samples and in most melanoma cell lines. Among normal cells, only melanocytes appear to express the gene. The tyrosinase antigen presented by HLA-A2 may therefore constitute a useful target for specific immunotherapy of melanoma. But possible adverse effects of antityrosinase immunization, such as the destruction of normal melanocytes and its consequences, will have to be examined before clinical pilot studies can be undertaken.

When blood lymphocytes of melanoma patients are stimulated in vitro with tumor cells of the same patient, one often observes the proliferation of T lymphocytes that exert cytolytic activity on the autologous melanoma cells (1–3). From these responder cell populations, it is often possible to isolate by limiting dilution cytolytic T lymphocyte (CTL)[1] clones that display a high activity on the tumor cells and do not lyse autologous EBV-transformed lymphoblastoid cell lines, fibroblasts, or NK targets such as K562 (3–6). With such antitumor CTL clones it is possible to select in vitro antigen-loss tumor cell variants. This approach indicated that melanoma cell lines display several different antigens recognized by autologous CTL (7, 8).

By transfecting into an antigen-loss variant a cosmid library prepared with the DNA of melanoma cell line MZ2-MEL, we obtained transfectants that expressed an antigen recognized by an autologous CTL clone (9). This led to the identification of gene MAGE-1, which directs the expression of antigen MZ2-E (10). Gene MAGE-1 is expressed in ~40% of melanoma tumor samples. It is also expressed on some other tumor types, such as lung and breast carcinoma (11). No expression was found in a large panel of normal tissues, with the exception of testis (F. Brasseur, unpublished results). Antigen MZ2-E is presented by class I MHC molecule HLA-A1. The MAGE-1-encoded antigenic peptide has been identified as a nonapeptide (12). Melanoma cells of other HLA-A1 patients that express gene MAGE-1 are recognized by the anti-MZ2-E CTL of the original patient.

Because MAGE-1 is not expressed in most normal tissues, antigen MZ2-E may constitute a useful target for specific immunotherapy. But only 10% of melanoma patients express this antigen on their tumor, since this requires that they carry the HLA-A1 allele (as do 26% of caucasians) and that their tumor expresses MAGE-1 (as do 40% of melanomas). It is therefore important to identify additional genes that code for tumor rejection antigens. Those that direct the expression of antigens presented by HLA-A2 should be particularly useful since this allele is very frequent (49% of caucasians).

Several groups have derived from HLA-A2 patients CTL that lyse not only the autologous tumor cells but also a large proportion of the cell lines derived from the tumors of other HLA-A2 patients (13, 14). We have isolated such CTL clones from two melanoma patients, and we report here the identification of the gene that codes for their target antigen.

[1] *Abbreviation used in this paper:* CTL, cytolytic T lymphocyte.

Materials and Methods

Patients and Cell Lines. In 1987, patient LB24 developed a malignant melanoma on the lower left leg cured by a large excision. She relapsed in 1988 with three metastatic skin nodules above and below the primary location. A half-calf amputation was performed. The patient is presently disease free. Tumor cell line LB24-MEL was obtained by adapting cells from the amputation sample to tissue culture. The melanoma cells were cultured in Iscove medium (Gibco Laboratories, Grand Island, NY) containing 10% FCS, supplemented with L-arginine (116 mg/liter), L-asparagine (36 mg/liter), and L-glutamine (216 mg/liter).

Patient SK29(AV) had recurrent metastatic malignant melanoma from 1975 to 1978. Melanoma cell line SK-MEL-29 was initiated from a metastatic lesion in 1976. We will refer to it as SK29-MEL (this change in nomenclature enables us to identify easily all cells coming from the same patient: SK29-MEL, SK29-CTL, . . .). SK29-MEL was a gift from Dr. Old (Memorial Sloan-Kettering Cancer Center, New York). SK29-MEL was cloned by limiting dilution and clone SK29-MEL.1 was selected for further experiments (6). Melanoma cell line NA8-MEL was a gift from Dr. F. Jotereau (Inserm U211, Nantes, France). The culture medium of SK29-MEL and NA8-MEL was DME (Gibco Laboratories) containing 10% FCS (Gibco Laboratories), 10 mM Hepes, and 4.5 g/liter glucose. The cells were cultured at 37°C in 8% CO_2.

Derivation and Culture of CTL Clones. Mixed lymphocyte tumor cultures (MLTC) of PBL and tumor cells isolated from patient LB24 were performed as previously described (3), except that the medium was supplemented with 5 U/ml of human rIL-4 (a gift from R. Devos, Roche Research, Gent, Belgium).

Derivation and long-term culture of CTL clones from PBL of patient SK29(AV) was previously described (8). The IVSB CTL line was derived from a MLTC responder cell population without cloning. However, eight subclones were isolated by limiting dilution and they were tested on all the autologous targets described in Results. All had exactly the same pattern of lysis. We therefore refer to this monospecific line as CTL clone IVSB.

Assay for Cytolytic Activity. The protocol was previously described (15). Target cells were treated for 48 h with 50 U/ml of human rIFN-γ (Boehringer, Ingelheim, Germany). CTL and 1,000 ^{51}Cr-labeled targets were incubated at various ratios in 96 conical microplates in a final volume of 200 μl. Chromium release in the supernatant was measured after 4 h of incubation.

Construction of the cDNA Library. Total RNA was isolated from clone SK29-MEL.1 and poly(A)$^+$ RNA was prepared by oligo(dT) binding (mRNA purification kit; Pharmacia Fine Chemicals, Piscataway, NJ). mRNA was converted to cDNA, ligated to EcoRI adaptors, and inserted into the EcoRI site of expression vector pcDNAI/Amp (Invitrogen Corporation, Oxon, U.K.) as described in the SuperScript plasmid system kit (Gibco BRL, Gaithersburg, MD). Recombinant plasmids were electroporated into JM101 *Escherichia coli* bacteria (Genepulser; Bio-Rad Laboratories, Richmond, CA) at 1 pulse at 25 μfarad and 2,500 V, that were selected with ampicillin (50 μg/ml).

Transfection of COS-7 Cells and Screening of Transfectants. DNA from pools of bacteria was transfected by the DEAE-dextran-chloroquine method into 3 × 10^4 COS-7 cells (16). 100 ng of plasmid pcDNAI/Amp-A2 and 100 ng of DNA of a pool of the cDNA library were used. Plasmid pcDNAI/Amp-A2 contains the HLA-A2 gene isolated from a CTL clone of patient SK29(AV). The COS cells were incubated for 48 h at 37°C. The medium was then discarded and 1,000-2,000 CTL were added in 100 μl of Iscove medium containing 10% human serum. For CTL clone 210/9, the medium was supplemented with 25 U/ml IL-2 (a gift of Biogen, Geneva, Switzerland). After 24 h, the supernatant was collected and its TNF content was determined by its cytolytic effect on W13 cells as previously described (9).

Transfection of a Melanoma Cell Line. Melanoma NA8-MEL line was transfected by the calcium phosphate precipitation method, as described (9). Briefly, 7.5 × 10^5 cells were seeded in 4.5 ml of medium. 24 h later, they were transfected with 2 μg of plasmid pSVtkneoβ (17) and 20 μg of cDNA 123.B2. After 48 h, the cells were seeded in microcultures at 2,000 cells/well in 200 μl of medium containing 2 mg/ml of neomycin analogue G418 (Gibco Laboratories). NeoR colonies were selected for further experiments.

PCR Assays for Tyrosinase Expression. Isolation of total RNA from tumor samples was performed as described (18). RNA from melanocyte culture was a gift from Dr. Old. Reverse transcription was performed on 2 μg of total RNA with an oligo(dT) primer. cDNA corresponding to 100 ng of total RNA (10^4 cell equivalents) was amplified for 35 cycles by PCR with primers. These primers are located in different exons to exclude the amplification of genomic DNA. An aliquot of the PCR reaction was run on a 1% agarose gel stained with ethidium bromide. To ensure that the RNA were not degraded, the cDNA products were tested for the presence of both human β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

For quantitative expression measurements, cDNA was obtained as above. RNA obtained from the clone SK29-MEL.1 was included pure and serially diluted in each series of quantitative PCR. The number of PCR cycles was reduced to 22 so that a linear dilution curve of the standard was obtained. Trace amounts of labeled dCTP (0.2 μCi) were added and accurate quantitation was obtained using the phosphor-imaging technology (Phosphor-Imager; Molecular Dynamics, Sunnyvale, CA). Expression of the various samples were normalized for RNA integrity by taking into account the expression level of the β-actin gene. When they showed values above that found with SK29-MEL RNA, the samples were evaluated again after dilution.

Results

An Antigen Recognized on HLA-A2 Melanoma Cells by CTL Clones of Two Patients. By stimulating blood lymphocytes of HLA-A2 patients SK29(AV) and LB24 with irradiated cells of autologous melanoma lines SK29-MEL and LB24-MEL, respectively, responder lymphocytes were obtained that lysed the autologous tumor cells. CTL clone IVSB was isolated from responder cells of patient SK29(AV), and CTL clone 210/9 was isolated from those of patient LB24. These CTL clones displayed high lytic activity on the autologous tumor cell lines (Fig. 1).

Both CTL showed broad reactivity on HLA-A2 melanoma cells. First, they lysed the melanoma cell lines of both patients. Further, they recognized most but not all melanoma cell lines derived from HLA-A2 patients (Fig. 1). Definite evidence that their target antigens were presented by HLA-A2 was provided by a variant of SK29-MEL that had been selected in vitro for resistance to another antitumor CTL clone of patient SK29(AV). This variant had lost the expression of HLA-A2. It proved resistant to CTL clones IVSB and 210/9 (Fig. 1). When the HLA-A2 gene was reintroduced by trans-

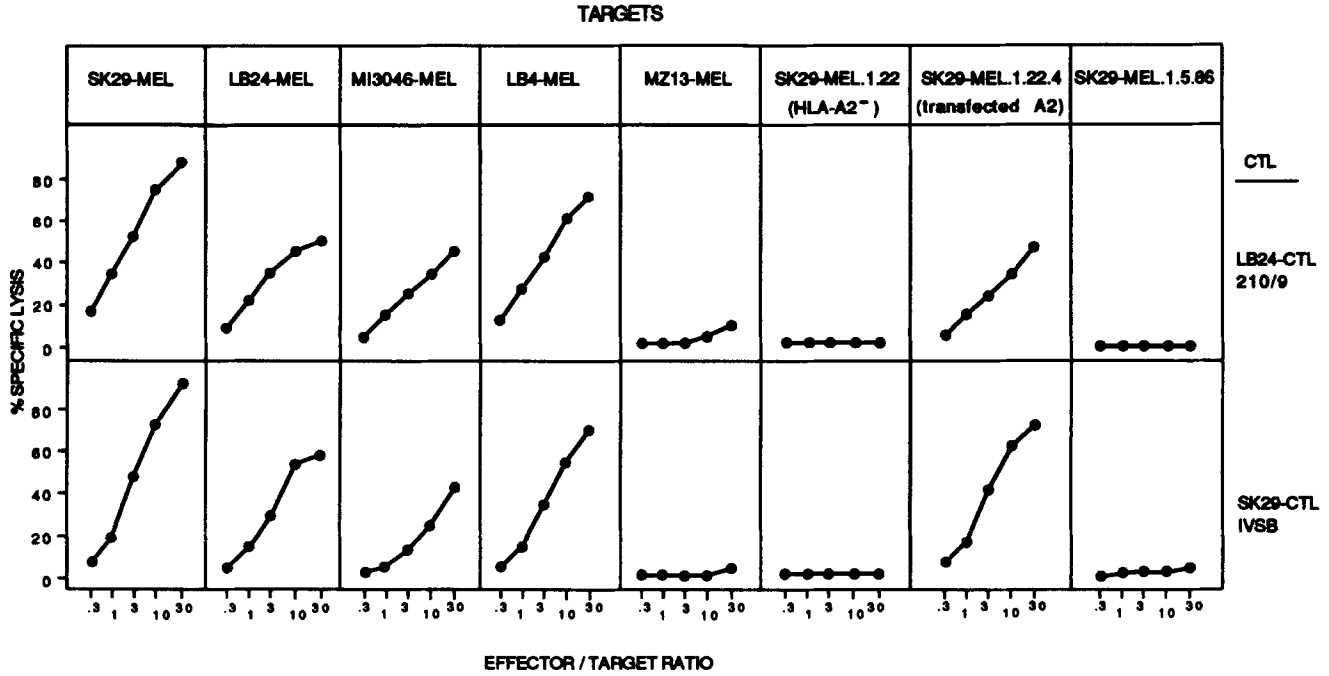

Figure 1. Sensitivity of melanoma target cells to lysis by CTL clones 210/9 and IVSB. Lysis of chromium-labeled cells was measured after 4 h. Melanoma cell lines SK29, LB24, MI3046, LB4, and MZ13 are derived from HLA-A2 patients. SK29-MEL.1.22 is a variant of SK29-MEL selected in vitro with an autologous CTL clone resulting in the loss of HLA-A2 (8). SK29-MEL.1.22.4 is the same variant transfected with the HLA-A2 gene of patient SK29. SK29-MEL1.5.86 is an antigen-loss variant of SK29-MEL selected in vitro for resistance to CTL IVSB (30).

fection into this variant, it regained sensitivity to both CTL (Fig. 1).

Evidence that CTL IVSB and 210/9 recognize the same antigen was provided by another antigen-loss variant of SK29-MEL. This variant, named SK29-MEL1.5.86, had been selected for resistance to CTL IVSB and had kept the expression of HLA-A2. It also proved resistant to lysis by CTL 210/9 (Fig. 1). The antigen, which was common to SK29-MEL and LB24-MEL and was recognized by autologous CTL clones IVSB and 210/9, was named SK29-Ab or LB24-Ab. We will refer to it as Ab.

Cloning of a cDNA Expressing Antigen Ab. We set out to identify the gene coding for antigen Ab by transfecting an expression library in COS cells (16). A cDNA library was prepared from RNA of a clonal subline of melanoma cell line SK29-MEL and it was cloned into plasmid pcDNAI/Amp. Bacteria were transformed with the products of ligation and the library was divided into 700 pools of 200 bacteria. Each pool contained ~100 different cDNAs because only 50% of the plasmids were found to carry an insert. Plasmid DNA was prepared from each pool and was then cotransfected into COS-7 cells together with the HLA-A2 gene of patient SK29(AV) cloned into pcDNAI/Amp. After 48 h, the transfected cells were tested for expression of antigen Ab by their ability to cause TNF release by the relevant CTL (9). The amount of TNF released by the CTL was measured by the cytotoxic effect of the culture supernatant on Wehi-13 cells.

Each cDNA pool was transfected in duplicate COS microcultures. One microculture was tested with CTL 210/9 and the other with CTL IVSB. For the 700 microcultures tested with CTL IVSB, all supernatants contained between 0.6 and 4 pg/ml of TNF, except for four that contained between 10 and 20 pg/ml. The four duplicate cultures also clearly stood out among those tested with CTL 210/9 as having produced more TNF than the others. Two of the four corresponding pools of bacteria were subcloned and plasmid DNA was extracted from each of ~500 bacteria. The DNA of these plasmids was transfected into COS cells and the transfectants were tested for their ability to stimulate CTL 210/9 and IVSB. One positive cDNA clone was found in each pool. The very effective stimulation of CTL clone 210/9 by COS cells transfected with cDNA clone 123.B2 is displayed in Fig. 2. This stimulation appeared to be specific: another A2-restricted CTL of patient SK29(AV), which was known to recognize another antigen, was not stimulated by the transfected COS cells (data not shown).

To confirm the results obtained with the transient COS cell transfectants, we used HLA-A2 melanoma cell line NA8-MEL, which was not recognized by anti-Ab CTL 210/9 or IVSB. This cell was cotransfected with the pcDNAI/Amp construct containing cDNA 123.B2 and with a plasmid conferring resistance to geneticin. Geneticin-resistant stable transfectant clones were isolated. 7 of 15 proved sensitive to lysis by CTL 210/9 and IVSB (Fig. 3). They were not lysed by another A2-restricted CTL directed against another antigen.

Antigen Ab Is a Product of the Tyrosinase Gene. cDNA

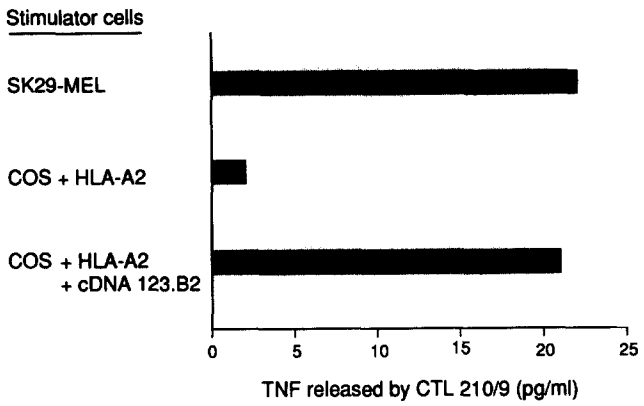

Figure 2. Stimulation of CTL 210/9 by COS cells transfected with pcDNAI/Amp constructs containing HLA-A2 and cDNA 123.B2 as described in Materials and Methods. Controls were transfected with HLA-A2 alone. CTL 210/9 was added and the supernatant was tested on Wehi-13 cells.

123.B2 was sequenced and found to be almost identical to a human tyrosinase cDNA previously isolated from a melanoma cell by Bouchard et al. (19). Both cDNAs contain an open reading frame of ~1,600 nucleotides that codes for the enzyme tyrosinase. Only three nucleotides differed between the two sequences, each difference resulting in an amino acid change. Two of the differences were located in exon 1 and one in exon 4 (Fig. 4). In two of three positions, our sequence was identical to another tyrosinase sequence reported previously (20).

Because of our previous observations that point mutations occurring on tumor cells can generate new antigens recognized by autologous CTL (21, 22), we compared the sequence of the tyrosinase cDNA obtained from the SK29 melanoma cells to that of the tyrosinase gene present in other cells of the same patient. We applied PCR amplification to the DNA of a CTL clone of patient SK29(AV) (Fig. 4). Five cloned

Table 1. *Quantitative Measurement of the Expression of the Tyrosinase Gene*

		Expression of tyrosinase (percent relative to SK29-MEL)
Melanoma cell lines		
	SK29-MEL.1	100
	LB39-MEL	280
	NA8-MEL	7
	LB24-MEL	760
	SK23-MEL	280
Melanoma tumor samples		
Primary tumors	LB357	88
	LG18	63
	LB373	150
Metastases	LB239	470
	LB15	97
	LB537	170
	LB435	620
	LB224	390
Melanocyte culture		530
Skin		
	Sample 1	0.1
	Sample 2	12
	Sample 3	35
	Sample 4	2
	Sample 5	25
	Naevus	170

cDNA synthesis and 22 cycles of PCR amplification were performed as described in Materials and Methods. Results were normalized according to the expression of β-actin. The results are expressed as the percentage of the level of expression of melanoma line SK29-MEL.

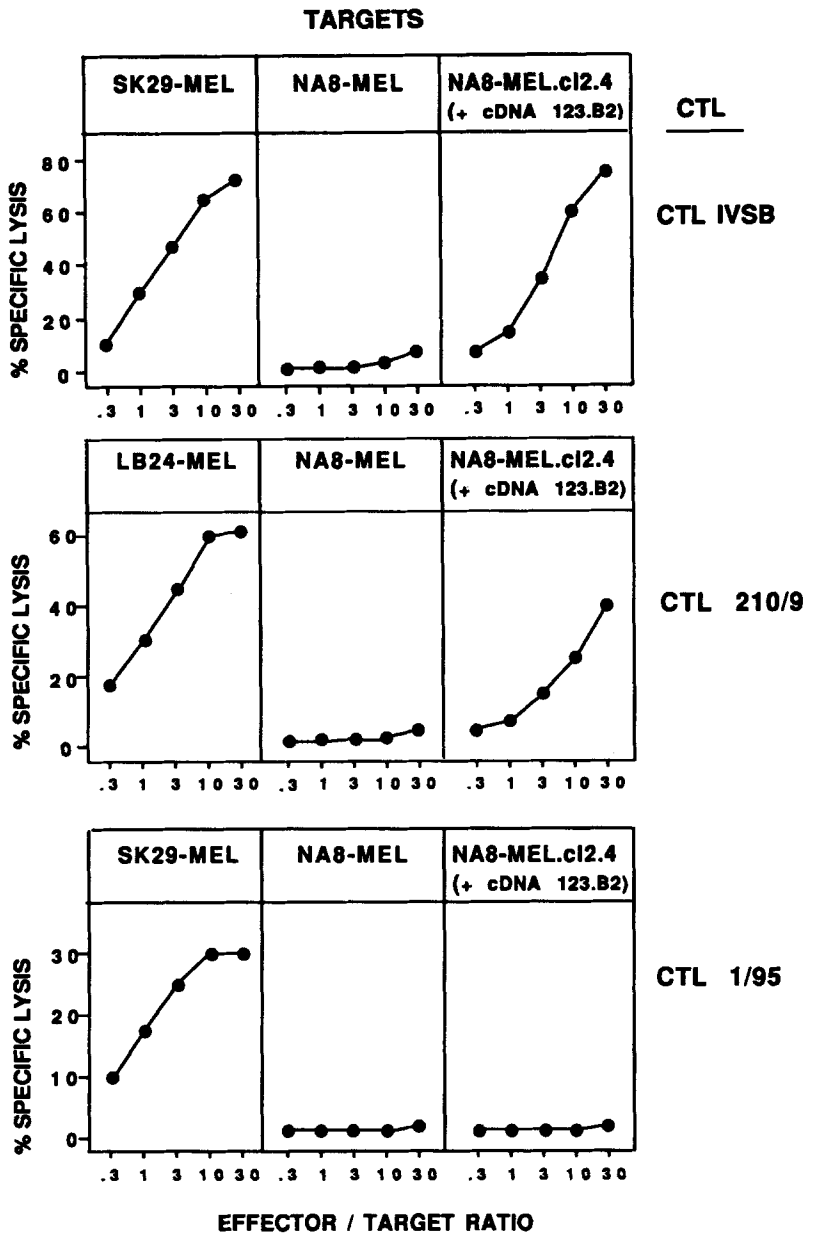

Figure 3. Expression of antigen Ab in HLA-A2 melanoma cell line NA8-MEL transfected with cDNA 123.B2. The cells were cotransfected with the pcDNAI/Amp-cDNA 123.B2 construct and pSVtkneoβ as described in Materials and Methods. Geneticin-resistant transfectant clone NA8.cl2.4 was tested for sensitivity to lysis by anti-Ab CTL IVSB and 210/9. CTL 1/95 is an A2-restricted CTL isolated from melanoma patient LB39 and directed against another antigen.

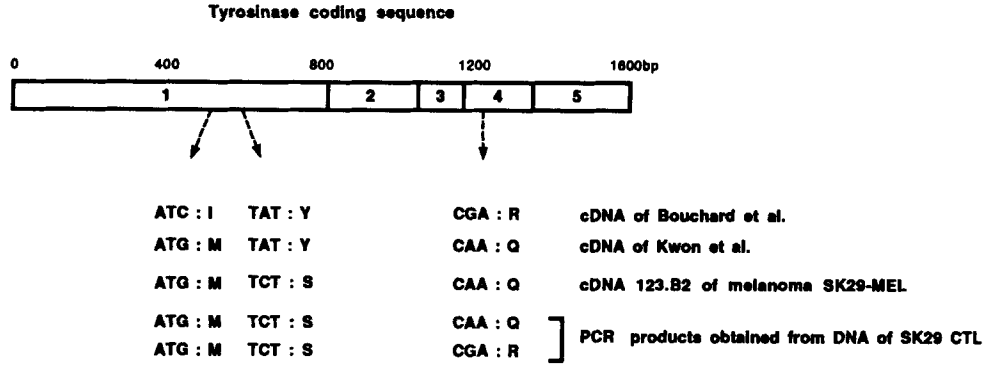

Figure 4. Codons and amino acids at the three positions (537, 575, 1205) where the sequence of cDNA 123.B2 and that of the cDNA clone isolated by Bouchard et al. (19) differ. The cDNA of Kwon et al. (20) differs from both of these sequences at a few other positions not shown here. The boundaries of exons 1–5 are indicated. Other explanations are given in the text.

products corresponding to exon 1 were sequenced; they were all identical to that derived from the melanoma cells. For exon 4, we obtained two different PCR products: one identical to that of cDNA 123.B2 and another identical to the sequence of Bouchard et al. (19). Thus, it appears that the three amino acid changes reflect a degree of polymorphism in the tyrosinase gene, and that patient SK29(AV) carries two alleles that differ at one position of exon 4. We conclude that the tyrosinase protein synthesized in the melanoma cells is identical to a product present in the normal melanocytes of patient SK29(AV).

Tyrosinase (monophenol,3,4-dihydroxyphenylalanine: oxygen oxydoreductase, E.C.1.14.18.1), which catalyses the synthesis of the melanin precursor dihydroxyphenylalanine (DOPA), has been reported to be specific for melanocytes. In adrenal cells and catecholinergic neurones, a similar reaction is catalyzed by tyrosine hydroxylase, an enzyme with an unrelated sequence (23). We verified the pattern of expression of tyrosinase by reverse transcription and PCR amplification of RNA prepared from various cells. All of 39 melanoma tumor samples were positive. Among melanoma cell lines we found 14 positives out of 21. No expression was found in tissues collected from heart, liver, kidney, lung, breast, ovary, prostate, testis, adrenals, and brain (including a substantia nigra sample). The level of expression found in melanoma samples ranged from 60 to 750% of that observed with the SK29-MEL cell line. A melanocyte culture displayed a level of expression that was about five times higher than that of SK29-MEL. Skin samples varied from 0.1 to 35% of the level observed with SK29-MEL (Table 1).

Discussion

The tyrosinase gene is the third gene that we have found to direct the expression of an antigen recognized on tumors by autologous CTL. The first gene was P1A, a gene expressed by mouse mastocytoma P815 (24). The second was human gene MAGE-1 (10). P1A and MAGE-1 have identical sequences in the tumor cells that express the relevant antigen and in autologous normal cells that do not. The presence of the antigen on the tumor cells is due to the expression of these genes, which are silent or nearly silent in most normal tissues. Presumably, this absence of expression in normal tissues allows for incomplete tolerance so that CTL against these antigens can be raised in vitro. Our evidence indicates that antigen SK29-Ab is also produced by a gene, tyrosinase, which is identical in the melanoma cells and in the normal cells of patient SK29(AV). We presume that melanocytes, the normal cells found to express the tyrosinase gene, also express antigen Ab, even though direct evidence is lacking.

Contrary to the antigen encoded by P1A and MAGE-1, the SK29-Ab antigen should be considered as a differentiation antigen since the gene is expressed specifically in melanocytes, the normal cell type from which the tumor is derived. It appears therefore that for differentiation antigens as well, tolerance is not absolute. However, it is possible that the high affinity lymphocytes have been eliminated by tolerance, and we may find that only CTL of relatively weak affinity can be obtained against such antigens. The observation that immune responses against differentiation antigens are not entirely suppressed by tolerance have been made previously with antibodies (25). Watanabe et al. (26) observed that some melanoma patients produced antibodies against ganglioside GD2, a structure that appears to be restricted to normal and malignant cells of neuroectodermal origin. Glycoprotein gp75, a very abundant intracellular glycoprotein expressed in pigmented human melanocytes and melanomas, was also recognized by antibodies present in the serum of a melanoma patient (27). This protein, which is the human homologue of the product of the mouse b (brown) locus, is also a differentiation antigen since it is specific for the cells of the melanocytic lineage (28).

Can a differentiation antigen like that encoded by tyrosinase serve as a useful target for immunotherapy? One argument in favor of this is provided by reports that destruction of melanocytes resulting in vitiligo is occasionally associated with melanoma regression and prolonged survival (29). Nevertheless, because melanocytes are present in tissues other than skin, one possible critical location being the choroid of the eye, it will be important to ascertain in a mouse model that T lymphocytes directed against tyrosinase antigens do not have unacceptable adverse effects on normal tissues.

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