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Immunology

Immunophenotyping of melanomas for tyrosinase: Implications for vaccine development

(tumor immunology/cancer vaccine)

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ABSTRACT **Tyrosinase (EC 1.14.18.1), the key enzyme in melanin synthesis, has been shown to be one of the targets for cytotoxic T-cell recognition in melanoma patients. To develop serological reagents useful for immunophenotyping melanoma for tyrosinase, human tyrosinase cDNA was expressed in an *Escherichia coli* expression vector. The purified recombinant tyrosinase was used to generate mouse monoclonal and rabbit polyclonal antibodies. The prototype monoclonal antibody, T311, recognized a cluster of protein moieties ranging from 70 to 80 kDa in tyrosinase mRNA-positive melanoma cell lines and melanoma specimens as well as in L cells transfected with tyrosinase cDNA. Untransfected L cells and L cells transfected with tyrosinase-related protein 1, TRP-1(gp75), were nonreactive. Immunohistochemical analysis of melanomas with T311 showed tyrosinase in melanotic and amelanotic variants, and tyrosinase expression correlated with the presence of tyrosinase mRNA. Melanocytes in skin stained with T311, whereas other normal tissues tested were negative. The expression pattern of three melanosome-associated proteins—tyrosinase, TRP-1(gp75), and gp100—in melanoma was also compared. Tyrosinase and gp100 are expressed in a higher percentage of melanomas than TRP-1(gp75), and the expression of these three antigens was discordant. Tyrosinase expression within individual tumor specimen is usually homogenous, distinctly different from the commonly observed heterogenous pattern of gp100 expression.**

Cutaneous melanin pigments are synthesized by melanocytes. The biosynthesis of melanin pigments involves a family of enzymes, including tyrosinase, tyrosinase-related-protein 1 (TRP-1), and tyrosinase-related-protein 2 (TRP-2) (1). Of these, tyrosinase (EC 1.14.18.1) is the principal enzyme, and mutations of the tyrosinase gene have been documented in various forms of albinism (2, 3).

The tyrosinase gene is composed of five exons, with the predicted amino acid sequence containing a leader signal peptide and a transmembrane domain (4, 5), consistent with it being a protein anchored to the membrane of the melanosomes. As a melanocyte differentiation antigen, tyrosinase is commonly expressed in malignant melanoma, as initially demonstrated by enzymatic 3,4-dihydroxyphenylalanine (dopa)-oxidase reaction (6) and subsequently by tyrosinase mRNA studies (7). Two recent reports indicated that tyrosinase is recognized by cytotoxic T cells from melanoma patients, one in the context of HLA-A2 (7) and the other in the context of HLA-A24 (8). The tyrosinase sequences recognized by HLA-A2-restricted cytotoxic T cells have been mapped to two nanopeptides, located in the signal peptide and in the catalytic domain (9). These findings open the way to immunotherapeutic strategies using tyrosinase peptides and/or protein. In this paper, we describe the production of recombinant tyrosinase protein and polyclonal and monoclonal antibodies (mAbs) that recognize tyrosinase. The frequency and characteristics of tyrosinase expression in melanomas were compared to the expression of two other melanosome-associated proteins, gp100 (10) and TRP-1(gp75) (11).

MATERIALS AND METHODS

Cell Lines and Tissues. Melanoma cell lines have been described previously (12–14), and cultured melanocytes were kindly provided by M. Eisinger (Lederle Laboratories, Pearl River, NY). L cells transfected with human tyrosinase or TRP-1(gp75) cDNA (15) were kindly provided by A. Houghton (Memorial Sloan–Kettering Cancer Center). Specimens of normal and tumor tissues were obtained from the Departments of Pathology at the New York Hospital–Cornell Medical Center and Memorial Sloan–Kettering Cancer Center.

mAbs. TA99, a mouse mAb against human gp75, has been described (11). HMB45, a mAb reactive with melanoma antigen gp100 (10), was obtained commercially (Dako).

Oligonucleotide Synthesis. Oligonucleotides were synthesized based on the published sequences (4) to amplify the tyrosinase gene. The 5′ primer, derived from exon 1, corresponds to the N-terminal sequence after cleavage of the signal peptide (5′-CACACGGATCCGATGACGATGACAAAGCCTGTGTCTCCTCTAAGAACC-3′, designated as tyrA). In addition to a *Bam*HI restriction site, this primer also contains the DNA sequences encoding an enterokinase cleavage site (Asp-Asp-Asp-Asp-Lys). Two 3′ primers were prepared, one derived from exon 5 (5′-CACACAAGCTTGATCCGACTCGCTTGTTCC-3′, designated as tyrB5), ending 5′ to the transmembrane domain sequences. The other 3′ primer was derived from exon 4 (5′-CACACAAGCTTGCCTTTGGAGCCACTGCTC-3′, tyrB4). Both 3′ primers include a *Hin*dIII restriction site for cloning. All oligonucleotides were synthesized commercially (Operon Technologies, Alameda, CA).

Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was prepared from melanoma cell lines or melanoma specimens, and tyrosinase mRNA expression was evaluated by RT-PCR using 35 PCR cycles and an annealing temperature of 60°C.

Prokaryotic Expression Cloning in *Escherichia coli*. PCR-amplified tyrosinase cDNA was cloned into expression vector pQE9 (Qiagen, Chatsworth, CA). Recombinant protein was induced by isopropyl β-D-thiogalactoside and purified by Ni^{2+} affinity chromatography, following the manufacturer's protocol (Qiagen). Protein was monitored by $NaDodSO_4$/polyacrylamide gel electrophoresis and silver staining.

Generation of Mouse Hybridomas and Rabbit Antisera. Purified recombinant tyrosinase was used to immunize

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Abbreviations: mAb, monoclonal antibody; RT-PCR, reverse transcription polymerase chain reaction; dopa, 3,4-dihydroxyphenylalanine.

BALB/c mice, and the spleen cells were fused with mouse myeloma cell line SP2/0. Hybridomas were generated and cloned as described (13), and screened by solid-phase ELISA using the immunizing fusion protein as the target antigen. A panel of melanoma antigens—e.g., MAGE-1 (16), expressed in the same vector—was used as a negative control for screening.

Antisera from rabbits immunized with the same recombinant protein were prepared by HRP (Denver, PA).

Immunoblotting. Immunoblotting was performed using a chemiluminescent detection system (Amersham) as described (16).

Immunohistochemical Procedures. The avidin-biotin complex immunoperoxidase procedure was carried out as described (17), using biotinylated secondary antibodies and avidin-biotin horseradish peroxidase complex. Diaminobenzidine tetrahydrochloride was used as the chromogen. In the case of pigmented tissues such as normal skin and melanomas, the secondary antibody step was followed by incubation with streptavidin-alkaline phosphatase conjugate (Boehringer Mannheim) and visualized with new fuchsin substrate. This second method generates a red reaction product, easily distinguishable from the brown-black melanin pigment. Isotype-matched unrelated immunoglobulins (Becton Dickinson) were used as negative controls. Immunofluorescent assays on transfected L cells were performed as described (18).

RESULTS

Typing of Melanoma Cell Lines and Melanoma Specimens for Tyrosinase mRNA. Twelve melanoma cell lines and 38 melanoma samples were evaluated for tyrosinase mRNA expression by RT-PCR amplification, using two sets of primer combinations—i.e., tyrA/tyrB4 and tyrA/tyrB5. Both primer pairs gave the same result. Of 12 melanoma cell lines, 7 (58%) were positive, and 5 were negative. Of 38 melanoma samples, 32 (84%) were positive, including grossly amelanotic melanomas. Four melanomas were negative, and 2 showed equivocal signals.

Production of Recombinant Tyrosinase Protein. Total RNA was prepared from cultured normal melanocytes. RT-PCR with tyrA/tyrB5 revealed the expected 1393-bp product, encoding 452 of the 511 amino acids in the mature tyrosinase molecule. This cDNA product was digested with *Bam*HI and *Hin*dIII and cloned into pQE9. The inserted tyrosinase sequence was confirmed by DNA sequencing. Isopropyl β-D-thiogalactoside induction of the transformed clones resulted in the synthesis of recombinant protein with an apparent molecular mass of ≈52 kDa, consistent with the predicted molecular mass. This recombinant tyrosinase contained hexahistidine at the N terminus and was purified by Ni^{2+} affinity column chromatography (Fig. 1).

Mouse mAbs. Mouse mAbs were generated against affinity-purified tyrA/tyrB5 recombinant protein and screened by ELISA. Clones secreting mAb showing reactivity toward recombinant tyrosinase but not against human MAGE-1 expressed in the same vector were harvested and subcloned. After three subclonings, seven clones were isolated—T41, T72, T125, T311, T550, T562, and T620. Each of the seven clones produced mAb with ELISA titer of 1:8000 to 1:32,000 against recombinant tyrosinase but no cross-reactivity with recombinant MAGE-1.

Rabbit Polyclonal Antisera Against Tyrosinase. The tyrA/tyrB5 recombinant protein was also used to immunize rabbits for polyclonal antisera. The polyclonal antisera showed the same reactivity pattern as the mouse mAbs by ELISA and immunoblotting analysis (Fig. 2*c*, see below).

Immunoblotting with Melanoma Cell Line Lysates. Nonidet P-40 lysates were prepared from SK-MEL-19, SK-MEL-30, and MZ2-MEL3.1. RT-PCR showed that SK-MEL-19 and SK-MEL-30 express tyrosinase mRNA, whereas MZ2-MEL3.1 does not. Immunoblotting with the seven mouse mAbs generated against tyrosinase showed a similar reactivity pattern, with the major antigenic species consisting of a cluster of proteins ranging from 70 to 80 kDa (Fig. 2*a*). A minor species of 55 kDa can be seen in cell lines that are strongly reactive—e.g., SK-MEL-19. T311, an IgG2a mAb, was selected as the prototype reagent because of its strong reactivity and was used in all subsequent experiments.

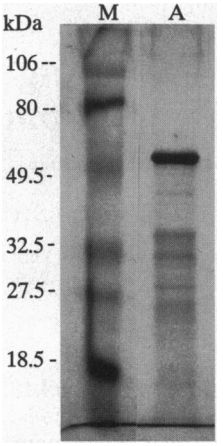

FIG. 1. Silver-stained gel of affinity-purified tyrA/tyrB5 tyrosinase recombinant protein (lane A), showing the main species at ≈52 kDa. Lane M, molecular mass standards.

Four additional cell lines were then tested with T311, including two tyrosinase mRNA-positive lines, SK-MEL-13 and SK-MEL-37, and two mRNA-negative lines, SK-MEL-187 and MZ2-MEL2.2. Results showed positive immunoblotting in the two mRNA-positive lines but not in mRNA-negative lines.

L Cells Expressing Tyrosinase React with T311. To prove that the protein species detected in immunoblotting were tyrosinase products, L cells transfected with the tyrosinase gene (15) were tested. Results showed that L-cell transfectants

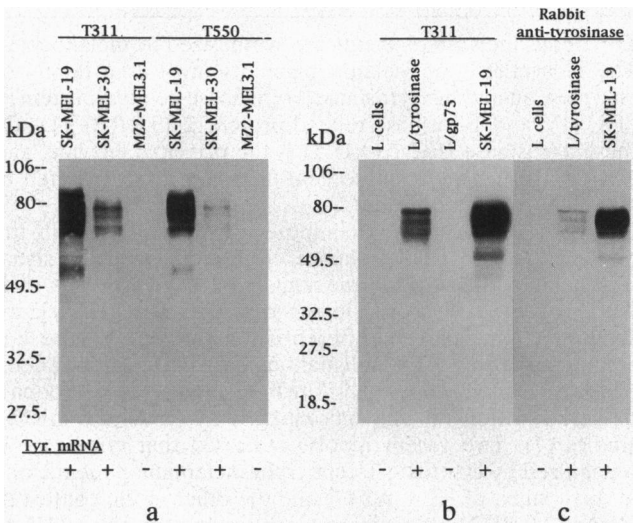

FIG. 2. Immunoblot analysis of anti-tyrosinase antibodies against cell line lysates correlated with tyrosinase mRNA expression by RT-PCR (bottom). (*a*) Two representative mAbs, T311 and T550, tested on three melanoma cell lines. The major reacting bands are in the range of 70–80 kDa, and a minor 55-kDa band is seen in SK-MEL-19. (*b*) L cells transfected with tyrosinase gene show a reactive pattern similar to tyrosinase-positive melanoma cell lines (e.g., SK-MEL-30 in *a*), whereas untransfected L cells and gp75-transfected L cells are negative. (*c*) Rabbit anti-tyrosinase polyclonal serum shows a similar reactivity pattern.

displayed an immunoblotting pattern similar to that of tyrosinase-positive melanoma cell lines (Fig. 2*b*)—i.e., a cluster of protein species at the range of 70–80 kDa. The 55-kDa species was not observed in the transfected L cells. Immunofluorescent assays with T311 showed positive reactivity in transfected L cells and no reactivity in the untransfected L cells.

T311 Does Not React with TRP-1(gp75). To rule out the possibility that T311 cross-reacts with TRP-1(gp75), L cells transfected with the TRP-1 (15) gene were tested. Immunofluorescent staining with TA99, a previously described anti-gp75 mAb, showed strong staining of these cells, confirming the expression of gp75 by L-cell transfectants. In contrast, immunofluorescent and immunoblotting assays of these transfectants with T311 were negative (Fig. 2*b*), indicating no cross-reactivity of T311 with TRP-1(gp75).

Immunohistochemical Reactivity of T311. T311 was tested against cytological preparations of SK-MEL-19, SK-MEL-187, and MZ2-MEL3.1 by the avidin-biotin complex immunoperoxidase procedure. Results showed positive cytoplasmic staining in the tyrosinase mRNA-positive line, SK-MEL-19, but not in the tyrosinase mRNA-negative lines. Strongest staining was observed in the perinuclear region, with the dendritic processes showing weaker reactivity. Frozen sections of normal skin from Caucasian and African-American individuals were then tested. Melanocytes from both specimens showed intense cytoplasmic staining, whereas pigmented basal keratinocytes and other cell types were negative (Fig. 3*a*). All noncutaneous normal human tissues tested were negative, including cerebral cortex, cerebellum, lung, kidney, spleen, colon, stomach, uterus, testis, skeletal muscle, smooth muscle, and adipose tissue. Normal retinal tissue, known to express tyrosinase (7), was unavailable for evaluation.

A panel of 16 melanoma specimens was tested, 13 tyrosinase mRNA-positive and 3 tyrosinase mRNA-negative, as defined by RT-PCR. Of the 13 tyrosinase mRNA-positive cases, 11 were amelanotic based on evaluation of the hematoxylin/eosin-stained sections. Eleven of 13 tyrosinase mRNA-positive cases showed positive cytoplasmic staining (Fig. 3*b*), whereas the three tyrosinase mRNA-negative cases were negative for T311 staining (Table 1). The two mRNA-positive, T311-nonreactive cases were found to have low or equivocal levels of tyrosinase mRNA.

Expression of Tyrosinase, TRP-1(gp75), and gp100 in Melanomas. With the same panel of 16 melanomas (see above), tyrosinase expression was compared to expression of two other melanocyte antigens, namely TRP-1(gp75) (detected by TA99) and gp100 (detected by mAb HMB45) (Table 1). TRP-1(gp75) was expressed in 8, tyrosinase in 11, and gp100 in 12 of the 16 cases. Detailed comparison of the cases indicated that the expression of these three antigens was discordant. The disso-

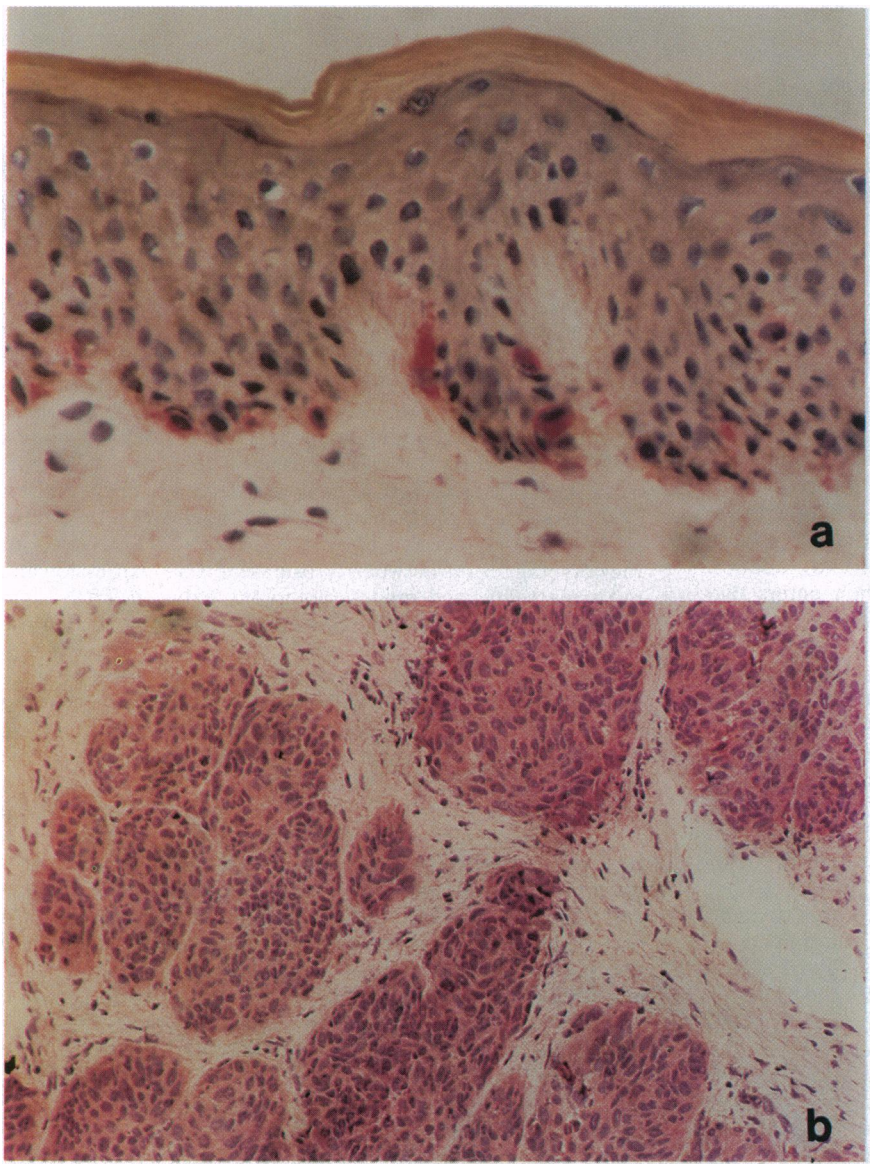

FIG. 3. Immunohistochemical reactivity of T311 with normal skin (*a*) and a melanoma specimen (*b*). Normal skin shows intense staining of the melanocytes (×360), and the melanoma specimen shows positive homogenous staining of the tumor cells (×180) (red, with new fuchsin substrate).

Table 1. Expression of tyrosinase, TRP-1, and gp100 in 16 melanoma specimens

Specimen	Tyrosinase		TRP-1	gp100
	mRNA*	T311†	TA99†	HMB45†
1	+	++	+++	+/++‡
2	−	−	−	−
3	+	−	−	−
4	+	++	+++	+++
5	+++	++	−/++	(−/+)
6	+	+	(−/++)	+++
7	+++	+/++	−/+	+++
8	+++	+++	++/+++	++
9	+++	+++	−	(−/++)
10	+++	++	−	(−/+)
11	−	−	−	−
12	−	−	−	−
13	+++	+	−	+++
14	+	−	+++	(−/++)
15	+++	+++	−	+++
16	+++	+++	++/+++	+++

Parentheses indicate cases with <20% of tumor cells stained.
*mRNA expression was determined by the quantity of RT-PCR products, judged by the intensities in ethidium bromide-stained gels.
†Antigenic expression was scored as −, +, ++, and +++ subjectively, based on the staining intensity.
‡Intratumor staining heterogeneity is indicated by two different scores in one entry.

ciation between TRP-1(gp75) and tyrosinase expression (four cases $TA99^{-}T311^{+}$ and one case $TA99^{+}T311^{-}$) adds further proof that T311 does not react with TRP-1(gp75).

Another significant difference concerns the heterogeneity of antigenic expression within individual tumor specimen. In contrast to tyrosinase-positive cases, where the staining intensity appears to be uniform among tumor cells, staining with HMB45 and TA99 was more heterogenous. This intratumor heterogeneity is particularly apparent in gp100 expression, as one-third (4 of 12) of the $HMB45^{+}$ cases showed positive staining in <20% of the tumor cells.

DISCUSSION

Two types of melanin pigments are produced by melanocytes—i.e., the black-brown eumelanin and the red-yellow pheomelanin (1). Tyrosinase is the key enzyme in the synthesis of both pigments, catalyzing two initial steps in the biosynthetic pathway—i.e., hydroxylation of tyrosine to dopa and oxidation from dopa to dopaquinone. Dopaquinone then enters two separate pathways, leading to the synthesis of eumelanin or pheomelanin. In addition to tyrosinase, several other structurally related proteins are encoded by the tyrosinase gene family, notably tyrosinase-related protein 1 (TRP-1) (19, 20) and tyrosinase-related protein 2 (TRP-2) (21–23). These two molecules are also involved in the melanin synthesis pathway, both of them contributing more to the synthesis of eumelanin than to pheomelanin (24–26). TRP-1 and TRP-2 share moderate sequence similarities with tyrosinase, the amino acid homology with the tyrosinase being 43% and 40%, respectively (22, 25).

The molecular mass of tyrosinase has been reported to range from 60 kDa (27) to 75 kDa (25, 28), as determined by enzyme-based assays in the earlier literature (29) and subsequently by immunoprecipitation or immunoblotting methods using antibodies (27, 28, 30). The predicted molecular mass of the primary translation product of the tyrosinase gene is ≈58 kDa (4, 31), which, after processing and glycosylation, leads to the mature tyrosinase molecules in the melanosomes, with a microheterogenous mass of 70–75 kDa. Variation in the size of the mature tyrosinase product has been attributed to the presence of isozymic forms (29) and/or alternate splicing of tyrosinase mRNA (31–33). With the anti-tyrosinase mAb T311 generated in the present study, we detected a cluster of proteins at the 70- to 80-kDa range, consisting of at least three or four species. These species are clearly encoded by the tyrosinase gene because transfection of L-cell fibroblasts with the human tyrosinase gene led to their expression. The detection of these multiple tyrosinase species by T311 is likely due to the fact that our series of tyrosinase mAbs was generated against the unglycosylated peptide backbone synthesized in *E. coli*, and the recognized antigenic epitope would therefore be present in mature tyrosinase as well as in precursor and intermediate forms. In accord with this idea, a minor 55-kDa product was present in cell lines expressing higher levels of tyrosinase, possibly representing the primary unglycosylated translation product.

Antibodies against mammalian tyrosinase have previously been produced, including polyclonal antibodies against hamster tyrosinase (34), mouse tyrosinase (30), and human tyrosinase (15). Two mAbs have also been described, against mouse T_4 tyrosinase (30) and human tyrosinase (27). mAb 5C12, described by McEwan *et al.* (27), was generated by immunization with a fraction of a human melanoma cell line lysate enriched for tyrosinase activity and was reported to recognize an antigenic epitope residing in the carbohydrate moiety of tyrosinase. A protein species of ≈60 kDa with tyrosinase activity was immunoprecipitated by 5C12, but an additional 70-kDa species with tyrosinase activity was not recognized by 5C12. The reactivity of 5C12 for tyrosinase-related proteins is not known, and no immunohistochemical analysis of its reactivity with melanoma has been reported.

The conclusion that T311, the mAb generated in this study, is specific for the tyrosinase gene product comes from several lines of evidence. (*i*) T311 reactivity with melanoma cell lines and melanoma specimens cotypes with tyrosinase mRNA expression. (*ii*) T311 shows strong positive staining of melanocytes in immunohistochemistry but does not react with pigmented keratinocytes or other normal tissues tested. (*iii*) T311 immunoreacts with the same spectrum of proteins from L cells transfected with the tyrosinase gene as it does from tyrosinase-expressing melanoma cells. Cross-reactivity of T311 with TRP-1 products was ruled out for the following reasons. (*i*) T311 reactivity in melanoma specimens does not cotype with TRP-1 expression (as determined by mAb TA99). (*ii*) T311 does not react with L cells transfected with the TRP-1 gene. Because of the lack of suitable reagents, we are unable at present to exclude reactivity of T311 with TRP-2. However, the cotyping results between tyrosinase mRNA expression and mAb reactivity, and modest homology between TRP-2 and tyrosinase (40%), argue against the likelihood of such cross-reactivity.

Because of the recent demonstration that tyrosinase gene products can be recognized by cytotoxic T cells (7, 8) and helper T cells (35) in humans, there is considerable interest in tyrosinase as an antigenic target for melanoma vaccines. Due to variation in the level of tyrosinase expression in melanomas from different patients, it would be important to define a standard typing method for tyrosinase. Three approaches can be considered—one by histologic evaluation of pigment production in tumor cells, the second by analyzing tyrosinase mRNA expression by RT-PCR, and the third by immunohistochemical staining with anti-tyrosinase antibody. Of these methods, evaluation of pigment production is the least reliable, since melanomas producing only pheomelanin will often be considered amelanotic, and it is indeed well known to pathologists that melanin pigments have been commonly found in amelanotic lesions by staining with ammoniated silver nitrate, such as the Fontana–Masson stain (6). Our present study further proves this point by demonstrating that most amelanotic tumors are positive for tyrosinase mRNA and protein.

The second method, typing tyrosinase expression at the RNA level, although sensitive, may be suboptimal for clinical specimens for the following reasons: (*i*) mRNA expression may not consistently correlate with protein expression, (*ii*) the possible heterogeneity of tyrosinase expression within individual tumors cannot be evaluated by RT-PCR assay, and (*iii*) RT-PCR may not reliably assess the levels of tyrosinase mRNA expression in melanoma, due to the limited reliability of RT-PCR in quantitative assays as well as the dilutional effect of RNA from adjacent nonneoplastic tissues. In comparison, immunohistochemical phenotyping with T311 provides a simple reliable alternative for evaluating tyrosinase expression in a semiquantitative fashion. Analysis of a series of melanomas revealed T311 staining in all tyrosinase mRNA-positive cases, with the exception of two cases that showed low tyrosinase mRNA expression and no T311 reactivity, presumably reflecting low antigen expression. Although this result indicated that T311 staining was not as sensitive as RT-PCR, it could also be argued that tumors expressing antigen in such low density may not be an effective target for tumor vaccination.

With regard to selecting patients for vaccine trials, it is clear that immunophenotyping melanomas for antigens such as tyrosinase gives no direct information about presence or level of T-cell-recognized peptides presented on the cell surface. However, it seems reasonable to assume that high homogenous expression of antigen is a desirable characteristic and that antigen expression would likely correlate with levels of presenting peptides. The value of this immunophenotyping approach becomes evident as we compare results with other antigens that are potential vaccine targets. In addition to tyrosinase, six other melanoma antigens have been shown to be recognized by host cytotoxic T cells—i.e., MAGE-1 (36), MAGE-3 (37), Melan-A/MART-1 (38, 39), gp100 (40), TRP-1(gp75) (41), and BAGE-1 (42), and mAbs are now available for three of them—namely, MAGE-1 (MA454, ref. 16 and 77B, ref. 43), gp100 (HMB45), and TRP-1(gp75) (TA99). The MAGE-1 mAbs have not been shown to be useful for immunohistochemical typing of melanomas. Comparison of the protein expression patterns of tyrosinase, TRP-1(gp75), and gp100 in melanomas reveals two important differences. First, tyrosinase and gp100 were expressed by a high percentage of melanomas, including amelanotic variants. In comparison, TRP-1(gp75) expression was less frequent. This observation parallels the expression profile of their mRNA species (41). Second, tyrosinase expression appears to be homogenous within individual tumors, which is significantly different from the heterogenous expression of gp100, seen by us and others (10). The expression pattern of gp75 appears to be intermediate with regard to heterogeneity. These two features of tyrosinase expression—expression by many melanomas and a homogenous pattern of expression—suggest that tyrosinase may be a favorable antigenic target for tumor vaccination. In addition, given the heterogeneic expression of some melanocyte differentiation antigens in melanomas, as we demonstrate here, immunophenotyping of individual tumors will likely be a critical step in evaluating patients for tumor vaccination.

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