Antibody Responses to Melanoma/Melanocyte Autoantigens in Melanoma Patients

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Melanogenesis-related proteins play important roles in melanin synthesis and antigenicity of melanomas. Identification of highly expressed melanoma-associated antigens (MAA) that are immunogenic in humans will provide potential targets for cancer vaccines. Melanogenesis-related proteins have been shown to be MAA. Autoantibody responses to these MAA have been shown to react with melanoma cells and melanocytes, and suggested to play a role in controlling melanoma progression. To assess antibody responses to potential melanoma/melanocyte autoantigens, the open-reading frame sequences of tyrosinase, tyrosinase-related protein (TRP)-1, TRP-2, and melanoma-associated glycoprotein antigen family (gp100/pmel17) genes were cloned and expressed as recombinant proteins in *E. coli*. Purified recombinant antigens were employed to detect antibodies in sera of melanoma patients and normal healthy donors. By affinity enzyme-linked immunosorbent assay and western blotting, all recombinant antigens were shown to be antigenic. The main subclass of antibody response to these antigens was IgG. Most importantly this study demonstrated anti-TRP-2 and anti-gp100/pmel17 IgG responses in melanoma patients. Only one of 23 normal donors had an antibody response to the antigens tested. MAA-specific IgG antibodies in sera were assessed in melanoma patients (n = 23) pre- and post-polyvalent melanoma cell vaccine treatment. Polyvalent melanoma cell vaccine treatment enhanced anti-MAA antibody responses; however, only anti-TRP-2 and anti-gp100/pmel17 antibody response was enhanced. These studies suggest that four melanogenesis-related proteins are autoimmunogenic and can be used as potential targets for active-specific immunotherapy. ***Key words: autoimmunity/gp100/TRP-2/vaccine. J Invest Dermatol 111:662–667, 1998***

One of the major inherent characteristics of melanocytes and melanoma cells is the expression of the melanogenesis-related proteins (Orlow *et al*, 1995; del Marmol and Beermann, 1996). The major melanogenesis-related proteins that have been cloned and characterized are tyrosinase, tyrosinase-related protein (TRP)-1, TRP-2, and melanoma-associated glycoprotein antigen family (gp100/pmel17) (del Marmol and Beerman, 1996; Jackson, 1988; Kwon *et al*, 1991; Tsukamoto *et al*, 1992; Kwon, 1993; Adema *et al*, 1994; Kobayashi *et al*, 1994). The functional enzyme activity of tyrosinase, TRP-1, and TRP-2 in production of melanin has been described in the Mason-Raper melanogenesis pathway (Kwon, 1993). These three enzymes have been shown to have ≈40% amino acid homology to each other. The role of gp100/pmel17 in the melanogenesis pathway is not known but has been associated with the filamentous matrix of melanosomes (Kwon *et al*, 1991; Adema *et al*, 1994). Gp100 gene has been shown to be highly related to the pmel17 gene family. The difference between these two genes is the in-frame deletion of a 21 base pair sequence in gp100 (Adema *et al*, 1994). In this study we will refer to gp100 and pmel17 as one antigen (gp100/pmel17). The melanogenesis-related proteins are consistently expressed in most melanoma tissue biopsies and melanocytes (Sarantou *et al*, 1997). Several of the proteins have been of major interest because of their roles in differentiation, pigmentation, and antigenicity (Orlow *et al*, 1995; del Marmol and Beermann, 1996). Color coating in the murine system has been correlated to specific gene loci (Orlow *et al*, 1995). These gene loci have been suggested to be related to the human melanogenesis-related proteins: tyrosinase, *albino* locus; gp100/pmel17, *silver* locus; TRP-1, *brown* locus; and TRP-2, *slaty* locus (del Marmol and Beerman, 1996).

Tyrosinase, TRP-1, and gp100/pmel17 have all been demonstrated to be antigenic in humans (Mattes *et al*, 1983; Bakker *et al*, 1994; Song *et al*, 1994; Baharav *et al*, 1995; Spagnoli *et al*, 1995; Naftzger *et al*, 1996). The antigenicity of these melanogenesis-related proteins has led investigators to utilize them as target antigens in human cancer vaccines. Because these melanogenesis-related proteins are autoantigens, "educating" the host immune system to convert from a potential state of immune tolerance or suppression to an effective anti-melanoma immune response has been a major task. Human leukocyte antigen restricted cytotoxic T lymphocyte (CTL) responses have been reproducibly demonstrated with tyrosinase and gp100/pmel17 peptides in melanoma patients by several groups (Bakker *et al*, 1994; Brichard *et al*, 1993; Spagnoli *et al*, 1995). Recently CTL have been shown to recognize peptides of TRP-1 and TRP-2 (Wang *et al*, 1995, 1996).

Vitiligo is a depigmenting skin disease in which melanocytes of the epidermis are destroyed (Cui *et al*, 1992; Song *et al*, 1994). The disease occurs in various degrees from small depigmented patches of skin to very large areas of the body. Although there has been strong linkage of the presence of anti-melanocyte antigen antibodies in vitiligo

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Abbreviations: AJCC, American Joint Committee on Cancer; CTL, cytotoxic T lymphocytes; gp100/pmel17, melanoma associated glycoprotein antigen family; MAA, melanoma-associated antigen; MAGE, melanoma antigen; NED, no clinical evidence of disease; PMCV, polyvalent melanoma cell vaccine; TRP-1, tyrosinase-related protein-1; TRP-2, tyrosinase-related protein-2.

patients, the etiology of the disease is still uncertain. Autoantibody responses to tyrosinase, TRP-1, and TRP-2 have been demonstrated in vitiligo patients' sera (Mattes *et al*, 1983; Song *et al*, 1994; Baharav *et al*, 1995; Kemp *et al*, 1997). TRP-1, originally defined as gp75, was identified and well characterized using melanoma patients' sera (Mattes *et al*, 1983; Vijayasaradhi *et al*, 1990). No major studies on autoantibody responses to gp100/pmel17 or TRP-2 proteins in comparing melanoma and vitiligo patients' sera have been demonstrated to date. Evaluation of immune responses to melanoma/melanocyte autoantigens in melanoma patients is important in determining their roles in tumor progression and as potential target antigens in active-specific immunotherapy. Melanoma-associated hypopigmentation, a clinical observation similar to vitiligo, is observed in melanoma patients (Merimsky *et al*, 1996). The relation between the functional role of melanoma/melanocyte autoimmunity to that of controlling melanoma progression remains to be determined. Although there have been studies on immune responses to individual melanoma/melanocyte autoantigens, there have not been any major melanogenesis-related studies assessing multiple autoantigens in the same patient.

Antibody responses to melanoma-associated antigens (MAA) are suggested to play an important role in controlling melanoma progression (Jones *et al*, 1981; Bystryn *et al*, 1992; Morton *et al*, 1992; Livingston *et al*, 1994; Mittelman *et al*, 1994; Naftzger *et al*, 1996). In recent years, emphasis of analysis of MAA has been on CTL studies; however, studies on antibody responses in humans are limited. This is primarily due to the limited availability of purified native or recombinant whole MAA protein. Recently, we have demonstrated antibody responses to the melanoma antigen (MAGE)-1 using purified recombinant protein (Hoon *et al*, 1995b). The availability of recombinant MAA proteins provides a useful tool in assessing antibody responses to the whole antigen in patients. This analysis is particularly useful when the specific antigenic epitope on the MAA is not known, or when there are multiple antigenic epitopes. Isolation of natural occurring melanoma proteins from melanoma specimens is very laborious and logistically unpractical for routine assay analysis. To assess melanoma/melanocyte autoantigen antibody responses in melanoma patients, we cloned, expressed, and purified recombinant proteins of tyrosinase, TRP-1, TRP-2, and gp100/pmel17 in *E. coli*, and developed MAA-specific enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Patients and sera collection Sera were collected from healthy volunteer donors and melanoma patients. Polyvalent melanoma cell vaccine (PMCV) treated patients had not received any immunotherapy, chemotherapy, or radiotherapy within 30 d prior to the start of the PMCV treatment. None of the patients had received immunotherapy anytime prior to PMCV treatment. Melanoma patients' blood was drawn prior to PMCV treatment (day 0). Melanoma patients were treated with PMCV consisting of three well-defined allogeneic melanoma cell lines as previously described (Morton *et al*, 1992; Hoon *et al*, 1995b). Briefly, patients were given PMCV on day 0, weeks 2, 4, 8, 12, and 16, and then every 3–6 mo. Post-PMCV blood was collected from patients after 4–5 PMCV treatments (weeks 12–16). This time point during PMCV has previously been shown to be the optimal point for assessment of anti-protein MAA responses if they are going to occur and be elevated from pre-PMCV (Morton *et al*, 1992). Patients underwent routine clinical evaluation for recurrence of disease during this time period. Melanoma patients in the study were selected on the basis of sera availability and by the following criteria: American Joint Committee on Cancer (AJCC) stage III disease with no clinical evidence of disease (NED) at the start of PMCV and after 4–5 treatments. These patients survived five or more years after PMCV treatment and had no evidence of disease recurrence. Because clinical disease progression can affect antibody responses and the state of immune responses, we selected patients who were disease-free with favorable responses to PMCV. Blood was collected and centrifuged, and serum aliquoted and cryopreserved at −30°C until assayed as previously described (Hoon *et al*, 1995b).

Expression vectors of MAA recombinant proteins Total RNA was prepared from the established melanoma cell line M12, and reverse transcribed into cDNA as described previously (Hoon *et al*, 1995a; Doi *et al*, 1996). The cDNA of tyrosinase (1356 bp), TRP-1 (1509 bp), TRP-2 (1356 bp), and gp100/pmel17 (1899 bp) were amplified by polymerase chain reaction. Oligonucleotides used to amplify the genes of tyrosinase, TRP-1, TRP-2, and gp100 were synthesized based on GenBank sequences. The primer sets for individual MAA were synthesized as follows: Tyrosinase, 5′-AGCTGGATCCGCCTGTGTCTCCTCTAAGAACC-3′ and 5′-ATGCCTCGAGGATCCGACTCGCTTGTTCCA-3′; TRP-1, 5′-GGTTGAGCTCACAATTCCCAAGACAGTGTGCC-3′ and 5′-CCTTCTCGAGTATTCTTTCTTCAGCATAGCA-3′; TRP-2, 5′-AGCTGGATCCCAGGGTCAGTTCCCCCGAGTC-3′ and 5′-ATGCCTCGAGCTCTCAACACCCGGTTGGACC-3′; gp100/pmel17, 5′-GTGAGAGCTCATTGGCTGTGATAGGTGCTT-3′ and 5′-CAAACT CGAGGAAGATGCGGGGTAGACG-3′. The 5′ primers include *Bam*HI (tyrosinase and TRP-2) or *Sac*I restriction site (TRP-1 and gp100) and the 3′ primers contain *Xho*I restriction site for cloning into the expression vector. Then the cDNA of the MAA were digested with respective restriction enzymes and cloned into an expression vector pGEX/HIS, allowing the expression of GST fusion protein with a C-terminal 6xHis affinity tag after induction. The pGEX/HIS vector was reengineered from the pGEX-2T vector (Pharmacia, Piscataway, NJ) by inserting into the *Sma*I site a DNA fragment containing multiple cloning sites followed by a 6xHis tag.

Expression and purification of recombinant proteins The protein expression plasmids were transformed into *E. coli* strain BL21 or DH5α cells. Recombinant fusion proteins were induced by isopropyl-D-thiogalactoside and purified from bacterial lysates by affinity chromatography using Glutathione Sepharose 4B (Pharmacia, Piscataway, NJ). The GST fusion proteins of MAA were first bound to the affinity column and the recombinant proteins tyrosinase, TRP-1, TRP-2, and gp100/pmel17 were cleaved from the GST carrier by thrombin and then eluted from the column. The affinity purified recombinant proteins were further purified by preparative sodium dodecyl sulfate gel electrophoresis using a Model 491 Prep Cell (Bio-Rad, Hercules, CA) or through an anion exchange "Q" column using the Bio-Rad FPLC system according to the manufacturer's instructions.

Western blotting analysis of recombinant MAA proteins Recombinant MAA proteins purified by affinity column (Glutathione Sepharose 4B) followed by cleavage with thrombin were applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted on to Hybond ECL nitrocellulose membrane (Amersham, Chicago, IL). For the detection of polyhistidine tag, the blot was blocked by 5% blocking reagent (Amersham) in phosphate-buffered saline (PBS)-0.05% Tween followed by incubation with 1: 500 dilution anti-His_6-peroxidase (Boehringer, Indianapolis, IN) in PBS-Tween. The blot was developed by ECL western blotting detection reagent (Amersham) following the manufacturers' protocol. For the detection of tyrosinase, the blot was blocked by 2.5% bovine serum albumin (Sigma, St Louis, MO) in PBS-0.05% Tween followed by incubation with 1:200 dilution in PBS at room temperature for an hour. After washing three times with PBS-Tween, the blot was incubated with 1:200 anti-tyrosinase mouse monoclonal antibody (Vector Laboratories, Burlingame, CA) in PBS at room temperature for an hour. After washing with PBS-Tween, the blot was incubated with 1:1000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (γ-chain specific) (Boehringer) and then developed using 5-bromo-4-chloro-3-indolyl-1-phosphate and nitroblue tetrazolium (Promega, Madison, WI) according to the manufacturer's procedure.

Reverse transcriptase-polymerase chain reaction (RT-PCR) of PMCV cell lines PMCV cell lines A, B, and C, and melanoma cell line M12, were established and characterized at the JWCI as previously described (Morton *et al*, 1992; Sarantou *et al*, 1997). RNA preparation, the RT-PCR assay, and the oligonucleotide primers of tyrosinase, TRP-1, TRP-2, and gp100/pmel 17 used in the RT-PCR assay were performed as previously described (Doi *et al*, 1996; Sarantou *et al*, 1997). Porphobilinogen deaminase was used as a control housekeeping gene in the RT-PCR reaction. Equal amounts (0.5 μg) of RNA were used for each reaction.

Affinity ELISA Affinity ELISA with purified recombinant proteins tyrosinase, TRP-1, TRP-2, and gp100/pmel17 containing a 6xHis tag was performed as previously described (Okamoto *et al*, 1997). Recombinant proteins were incubated overnight at room temperature in Ni^{2+} chelate-coated ELISA microwell plates (Xenopore, Hawthorne, NJ) according to the manufacturer's procedure. Sera were added in 2-fold dilution with PBS from 1:40 to 1:1280 and incubated at room temperature for 2 h in duplicate. Goat anti-human γ-chain or human μ-chain antibody conjugates (Boehringer) were used for detection of human IgG or IgM specific antibodies, respectively. ELISA were developed and read using a Molecular Device ELISA reader and analyzed using software provided by the manufacturer (San Francisco, CA). Antibody titers were defined as the highest serum dilution yielding absorbency reading greater than the mean plus two standard deviations above normal donor volunteer serum background absorbance at 1:40 dilution. Although 1:1280 serum dilution was tested, no patient was positive for this titer.

Western blotting Western blotting was performed as previously described (Hoon *et al*, 1995b). Recombinant proteins were run on sodium dodecyl

sulfate-polyacrylamide gel electrophoresis and blotted. Patients' sera were diluted in PBS buffer, followed by incubation with 1:1000 dilution of alkaline phosphatase-conjugated anti-human IgG (γ-chain specific) or anti-human IgM (μ-chain specific) (Boehringer) and then developed by using 5-bromo-4-chloro-3-indolyl-1-phosphate and nitroblue tetrazolium (Promega) according to the manufacturer's procedure.

Statistical analysis Assessment of antibody responses between patients and normal donors was carried out using Wilcoxon rank test. Analysis of antibody responses pre-PMCV immunization *versus* post-PMCV immunization was carried out using a generalized McNemar test (Fleiss, 1981).

RESULTS

Cloning, expression, and purification of MAA recombinant proteins The MAA tyrosinase, TRP-1, TRP-2, and gp100/pmel17 genes open-reading frame were successfully cloned, expressed, and purified. The amplified cDNA of tyrosinase (1356 bp) and cDNA of TRP-2 (1356 bp) both code for N-terminal 452 amino acid proteins. The amplified cDNA of TRP-1 (1509 bp) code for the full-length protein 503 amino acids, and cDNA of gp100/pmel17 (1899 bp) code for the protein 633 amino acids. The cDNA products were subcloned into specific sites of the expression vector pGEX/HIS. The vector pGEX/HIS, containing multiple cloning sites flanked by GST gene at the 5′-end and 6xHis tag at the 3′-end, was constructed by replacing the *Sma*I site of pGEX-2T with a blunt-ended *EcoR* V-*Blp* I fragment from pET-30b(+) vector (Novagen, Madison, WI). Therefore, induction of the transformed clones by isopropyl-D-thiogalactoside resulted in the expression of a GST fusion protein with the 6xHis tag at the C-terminus. The expression of recombinant fusion proteins of the MAA was verified by western blot analysis by both anti-GST antibody and Ni^{2+} -NTA conjugate antibody.

The four recombinant GST fusion MAA proteins were purified by GST affinity column chromatography, and the MAA alone were cleaved from the GST peptide by thrombin protease and then eluted from the column. Western blotting analysis of the individual recombinant MAAs with 6xHis tag was detected using anti-polyhistidine antibody (**Fig 1*A***). Both recombinant tyrosinase and TRP-2 have a molecular mass of 54 kDa, which was similar to the predicted molecular mass of the translation product (estimated 53 kDa). Recombinant TRP-1 has a molecular mass of 63 kDa, which was slightly larger than the estimated molecular mass of 59 kDa. Gp100/pmel17 was 67 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, which was close to the estimated molecular mass (69 kDa). Differences in recombinant protein size as determined by gel electrophoresis compared with the predicted size is often observed (Hoon *et al*, 1995b). This difference by gel analysis can be due to protein charge, overall amino acid composition, and/or migration ability in the gel. Different preparations and purifications of the individual recombinant MAA showed similar sizes.

To further verify the validity of the recombinant MAA, anti-tyrosinase antibody was assessed in western blot analysis against the four MAA (**Fig 1*B***). Only tyrosinase protein reacted with the antibody at the molecular weight shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis.

MAA mRNA expression by PMCV lines The PMCV cell lines were assessed by RT-PCR for the expression of MAA (**Fig 2**). Normal melanocytes expressed all the MAA markers, and in general these MAA are frequently (>75%) expressed in primary and metastatic melanoma biopsies (Sarantou *et al*, 1997). All PMCV cell lines were RT-PCR positive for all MAA markers. As positive controls, we assessed individual MAA plasmid DNA (tyrosinase, TRP-1, TRP-2, and gp100/pmel17). The melanoma cell line M12 used to clone the MAA genes was shown to express all MAA by RT-PCR analysis.

Western blot analysis of anti-MAA responses Initial screening of melanoma patients antibody responses to MAA was performed by western blotting. Sera from melanoma patients and normal volunteer donors were assessed at various dilutions. Representative western blot analysis of anti-tyrosinase, TRP-1, TRP-2, and gp100/pmel17 IgG antibody responses are shown in **Fig 3**. Normal donors showed no

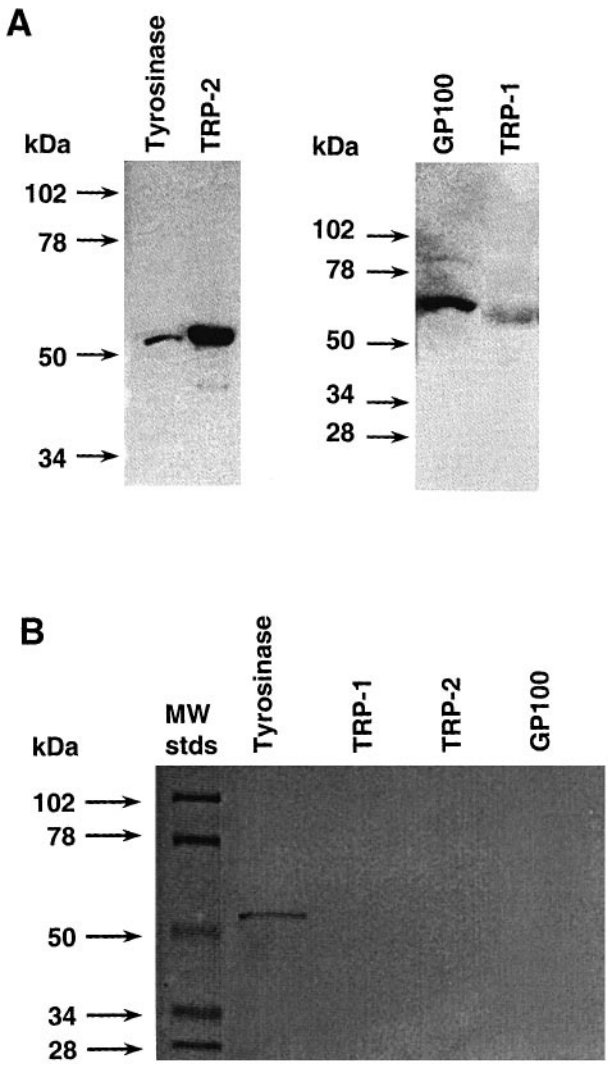

Figure 1. Western blotting analysis of recombinant MAA proteins detected by (*A*) anti-polyhistidine monoclonal antibody and (*B*) anti-tyrosinase antibody. All MAA were blotted on to the membranes in equal amounts.

MAA-specific IgG or IgM responses. Analysis of IgM responses to the individual MAA were very weak or absent in melanoma patients. Western blot analysis demonstrated antibody specific binding to the purified recombinant MAA. A control protein (HOJ-1 with a 6xHis tag) expressed as an *E. coli* recombinant fusion protein and purified in a similar manner as the MAA showed no antibody reactivity in western blots. Patients were also assessed for anti-MAGE-3 antibodies using MAGE-3 recombinant protein. MAGE-3 protein was purified in a similar manner to the other recombinant MAA. Anti-MAGE-3 IgG and IgM antibodies were found infrequently at low antibody dilutions (data not shown). This indicated that the binding of antibody to the melanogenesis autoantigens was not due to an expression induced artifact, nor a result of *E. coli* bacterial components potentially carried during the purification of the recombinant proteins nor due to reactivity to 6xHis tag. Differential antibody response to individual MAA from the same patients also indicated the responses were specific to the antigens.

Anti-MAA responses in melanoma patients and healthy normal donors An anti-MAA affinity ELISA was developed to assess melanoma patients' IgG responses. Recombinant protein with a 6xHis tag was coated on Ni^{2+} chelate-coated ELISA microwell plates. In previous studies by our laboratory and others, affinity ELISA using tagged recombinant proteins was more consistent and sensitive than conven-

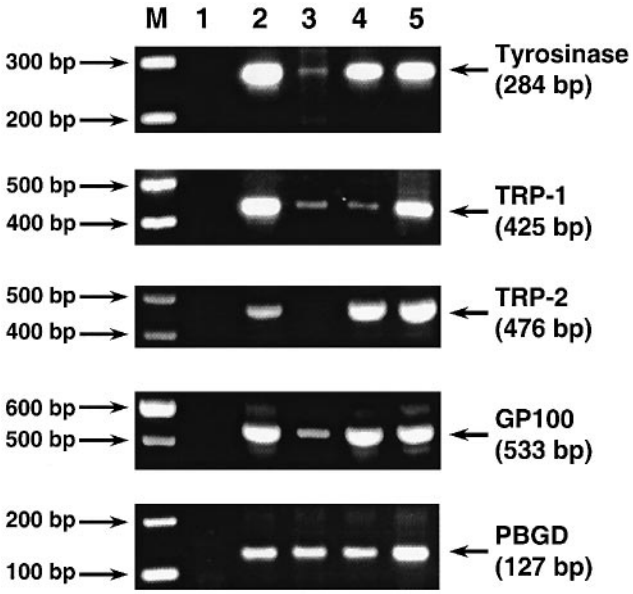

Figure 2. RT-PCR analysis of PMCV lines for expression of tyrosinase, TRP-1, TRP-2, and gp100/pmel17 mRNA. *Lanes 1–5* represent H_2O control, PMCV line A, PMCV line B, PMCV line C, and M12 control melanoma cell line, respectively. *Lane 3* (PMCV line B) for TRP-2 has a weak positive band not visible. *Lane M* refers to the base pair ladder as a reference.

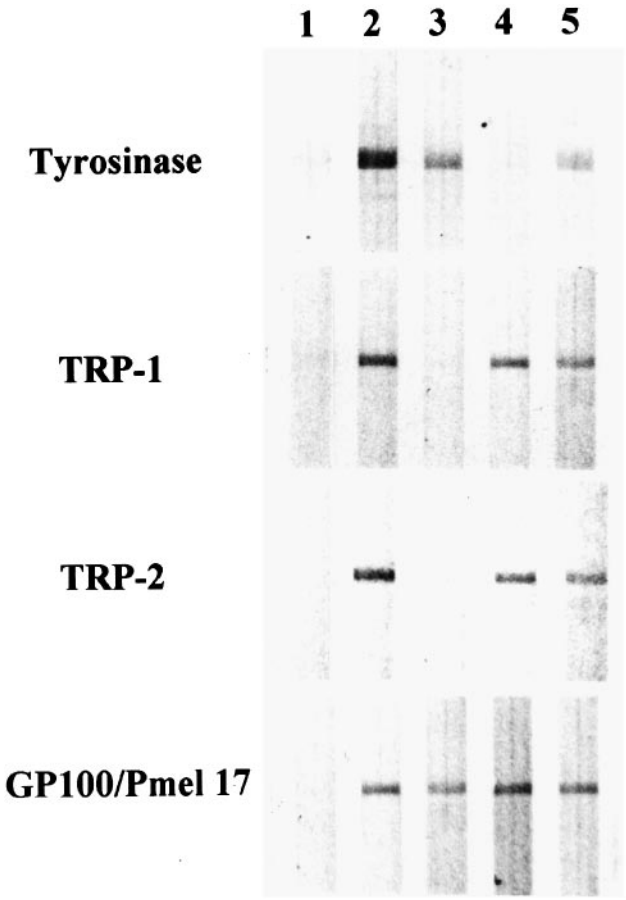

Figure 3. Representative western blot analysis of melanoma patient sera against recombinant tyrosinase, TRP-1, TRP-2, and gp100/pmel17 proteins. Representative examples of normal donor (*lane 1*) and four individual patients' (*lanes 2–5*) sera at a dilution 1:100 are shown.

tional protein adsorption ELISA (Okamoto *et al*, 1997). In this study only IgG were evaluated because western blot analysis of patients' sera to recombinant MAA had very weak or no IgM responses. Melanoma patients who were AJCC stage III NED and received PMCV were followed up for more than 5 y in the study. Selecting patients with a uniform, defined clinical status and equivalent follow-up was essential in evaluating immune responses to the recombinant autoantigens. The uniformity of patients' clinical status is very important in interpreting therapeutic efficacy of treatment protocols and also in the evaluation of antigen-specific immune responses in patients. Often, the latter criteria are ignored in immunologic assessments. Also, it was important that patients had no previous immunotherapy prior to the start of the PMCV therapy. This screening process is critical because both tumor burden and other treatment can potentially affect immune responses to MAA.

In comparing normal healthy donors *versus* pre-PMCV melanoma patients, significantly higher IgG responses were observed for all four MAA in the latter group (**Table I**). Only one normal donor had an antibody response (1:40) to three of the MAA. Normal donors and patients were matched on the basis of age and sex. This minimized any potential bias in normal *versus* patient comparisons. Antibody titers were measured in the range of 1:40–1:640 dilutions. The highest antibody titer was observed at 1:640 for TRP-2 and at 1:320 for tyrosinase, TRP-1, and TRP-2. The majority of the patients' anti-MAA antibody titers were clustered around 1:40 and 1:80. Assessment of antibody responses in nontreated patients showed overall similar results for individual patients. Non-treated patients with >1:40 antibody dilution against individual MAA were observed in nine of 23 (39%) patients against tyrosinase and five of 23 (22%) patients for the other three MAA (**Table I**). Patients' IgG titers (high and low responders) to individual MAA did not correlate to patients' human leukocyte antigen class I phenotype (data not shown).

Anti-MAA IgG responses after PMCV treatment Twenty-three melanoma patients (AJCC stage III, NED) who had received 4–5 PMCV treatments were evaluated for anti-MAA IgG responses. Two of the PMCV cell lines were positive using anti-gp100 murine monoclonal antibody (HMB-45) staining by flow cytometry. In **Fig 4** anti-MAA IgG titers pre- and post-PMCV immunization are shown. Patients were assessed at week 12–16 post-immunization (4–5 PMCV treatments). This period is based on observation of optimal antibody responses to other previously studied MAA in a PMCV-treated patient (Morton *et al*, 1992). Several of the positive patients' sera pre- and post-immunization were verified for anti-MAA specificity by western blotting to the individual MAA.

Enhancement of anti-MAA antibody responses after PMCV treatment was observed in patients (**Table I**). The most significant enhancement in antibody response post-immunization was observed against TRP-2 ($p = 0.01$) and gp100/pmel17 ($p = 0.016$) (**Table I**, **Fig 4*c*, *d***). Comparison of pre- *versus* post-PMCV anti-Trp-2 responses in seven patients showed an increase in titer. The post-PMCV analysis of anti-gp100/pme117 IgG responses showed an increase in titer in six patients. Enhancement of post-PMCV anti-tryosinase and anti-TRP-1 IgG responses were detected in four and three patients, respectively; however, no significant changes were observed in comparing pre-*versus* post-PMCV treatment for both MAA responses. In assessing patients with >1:40 titer post-immunization, anti-tyrosinase, and anti-Trp-2 was observed in 10 of 23 patients (44%), anti-gp100/pme117 in eight of 23 patients (35%), and anti-TRP-1 in four of 23 (17%) patients.

DISCUSSION

In this study we set out to examine antibody responses to melanogenesis MAA in normal healthy donors and melanoma patients. Using western blot analysis with recombinant MAA we demonstrated anti-MAA IgG antibody responses in melanoma patients. One of the novel features of the study was the demonstration of the immunogenicity of individual MAA and, in particular, TRP-2 in humans. All the MAA except TRP-2 have been demonstrated to be immunogenic in humans by either antibody or CTL assays. It is somewhat surprising that the major known enzymes in the melanogenesis pathway are all immunogenic

Table I. Anti-MAA IgG titers in normal donors and melanoma patients

Anti-MAA IgG	Source of sera[a]	Detection of anti-MAA at different dilutions[b] 1:40	1:80	1:160	1:320	1:640	Positive/total tested (%)
Tyrosinase	Normal donors	1	0	0	0	0	1/23 (4)
	Pre-PMCV	4	4	3	2	0	13/23 (57)
	Post-PMCV	3	3	5	2	0	13/23 (57)
TRP-1	Normal donors	0	0	0	0	0	0/23 (0)
	Pre-PMCV	5	4	1	0	0	10/23 (43)
	Post-PMCV	5	2	1	1	0	9/23 (39)
TRP-2	Normal donors	1	0	0	0	0	1/23 (4)
	Pre-PMCV	6	3	1	1	0	11/23 (48)
	Post-PMCV	3	6	2	1	1	13/23 (57)
Gp100/pmel17	Normal donors	1	0	0	0	0	1/23 (4)
	Pre-PMCV	6	3	2	0	0	11/23 (48)
	Post-PMCV	5	5	3	0	0	13/23 (57)

[a]Pre-PMCV sera was tested on day 0 of the first PMCV treatment. Post-PMCV sera were assessed after 12–16 wk of PMCV treatment.
[b]Normal donors *versus* pre-PMCV melanoma patients for anti-tyrosinase, anti-TRP-1, anti-TRP-2, anti-gp100/pmel17, were $p < 0.0001$, < 0.003, < 0.005, and < 0.005, respectively.

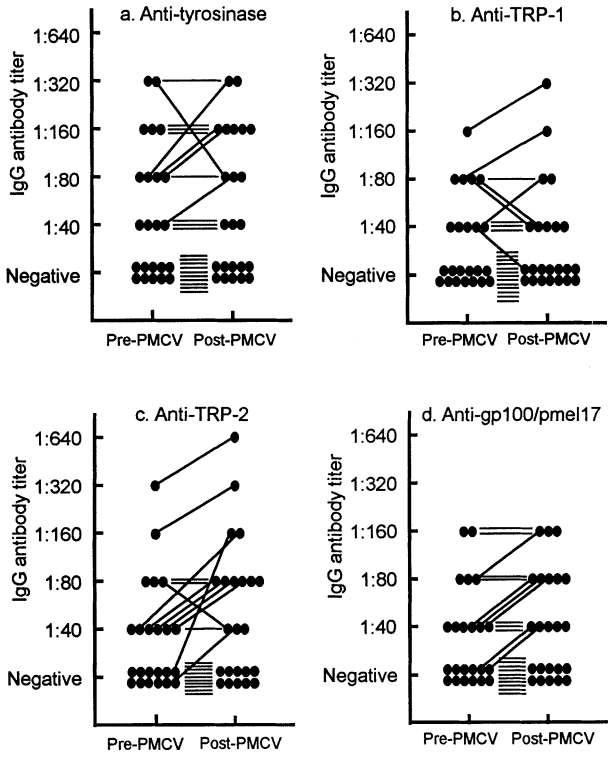

Figure 4. Assessment of PMCV-treated melanoma patients for induction of anti-MAA IgG responses. In individual patients' responses pre- and post-PMCV treatment are shown. Each point represents the mean of duplicate assays.

in humans. All of the melanogenesis-related MAA studied in this report are expressed in melanocytes (del Marmol and Beerman, 1996; Sarantou *et al*, 1997). The four melanogenesis MAA studied are expressed in greater than 75% of primary and metastatic melanoma biopsies from individual patients as assessed by RT-PCR plus Southern blot analysis (Sarantou *et al*, 1997). Antibody responses to all melanogenesis MAA were observed in melanoma patients and not in normal donors, with the exception of only one donor. Previously, TRP-1 and tyrosinase have been demonstrated antigenic by assessment of melanoma patients' sera (Mattes *et al*, 1983; Fishman *et al*, 1997).

Human TRP-2 gene was recently cloned and functionally identified as the enzyme dopachrome tautomerase responsible for the conversion of the dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (Tsukamoto *et al*, 1992; Bouchard *et al*, 1994). TRP-2 has been demonstrated to be more consistently present in melanotic or amelanotic melanomas; however, no direct correlation of eumelanin to TRP-2 expression and activity has been shown. We have demonstrated that TRP-2 mRNA is found in primary and metastatic melanoma biopsies (Sarantou *et al*, 1997). Recently, a TRP-2-derived peptide has been shown to be antigenic by recognition of human CTL (Wang *et al*, 1996). This study suggested the potential antigenicity of the TRP-2 gene product. Our analysis of TRP-2 protein in melanoma patients treated with active-specific immunotherapy with TRP-2 (PMCV) indicates that TRP-2 is immunogenic in humans. TRP-2 may be potentially useful as an immunogen similar to tyrosinase or gp100 in cancer vaccines. TRP-2 as well as TRP-1 have been identified to be intracellular proteins primarily associated with melanosomes (del Marmol and Beerman, 1996). TRP-1 has been shown to be expressed on the cell surface of melanomas (Takechi *et al*, 1996). Antibodies to other melanocyte antigens have been demonstrated in the autoimmune disease vitiligo but their specificities have not been well defined (Naughton *et al*, 1983; Cui *et al*, 1992).

Autoantibody responses to some of the individual melanogenesis MAA were elevated in some patients in post-PMCV compared with pre-PMCV sera. The anti-TRP-2 and gp100/pmel17 had the most significant responses post-immunization. Gp100/pmel17 has been shown to be very antigenic in humans, as demonstrated by CTL induced to gp100/pme117-derived peptides (Bakker *et al*, 1994; Spagnoli *et al*, 1995). The IgG response to gp100/pmel17 suggests that a T $helper_2$ response can also be induced. Currently, several cancer vaccines have focused on melanogenesis-related MAA such as gp100/pmel17 human leukocyte antigen-A2 motif peptides to induce T cell responses in patients (Maeurer *et al*, 1996; Zhai *et al*, 1996). To date, however, presence of anti-melanogenesis MAA T cell responses in immunized patients have not consistently been correlated to significant improved prognosis. Better strategies are needed in immunization and delivery. If a strong IgG response to an antigen is induced there is a potential for CTL or T $helper_1$ responses to be suppressed. This phenomenon has been referred to as "immune deviation" and documented for various *in vivo* antigen responses (Tuttosi and Bretscher, 1992). An immune deviation towards a particular type of response on immunization may not always be efficacious. Studies need to be designed to determine the significance of both antibody responses and CTL responses to specific MAA in melanoma patients.

The significance of the antigenicity of the individual melanogenesis MAA in melanoma patients is unknown. One of the most important observations in this study is that PMCV can enhance or induce IgG responses to melanogenesis MAA. Although the response did not occur in all patients, there was an indication that patients could be induced to produce higher titers to the individual melanogenesis MAA. The native proteins of MAA may be more antigenic and produce a higher anti-MAA response compared with denatured proteins. The immunologic events relating to immune self-recognition of melanocytes and melanoma rejection have been puzzling (Houghton, 1994). There is strong suggestive evidence that the destruction of melanocytes in vitiligo is due to autoantibody(s) against melanocytes that may be similar to mechanisms involved in immune-related melanoma regression

(Cui *et al*, 1992; Merimsky *et al*, 1996; Kawakami and Rosenberg, 1997). Several of the autoantibodies reactivities to specific melanocyte/melanoma antigens are well defined, whilst others are not. Anti-tyrosinase antibodies have been reported in vitiligo as well as melanoma patients (Song *et al*, 1994; Merimsky *et al*, 1996; Fishman *et al*, 1997). The immunologic responses to the specific melanogenesis-related proteins in vitiligo and melanoma patients with tumor regression after therapy have not been well studied. There is the possibility that the anti-melanogenesis MAA response is a secondary immunologic event as a consequence of the release of MAA after melanoma cell destruction from other events. Vitiligo depigmentation has been observed in several melanoma patients being treated with PMCV and in other immunotherapy studies reported (Berd *et al*, 1996; Kwakami, 1997).

The availability of recombinant MAA will allow us in the future to assess melanoma patients for antigen-specific antibody responses during active-specific immunotherapy. The induction of antibody responses in patients is an indicator that the immunization protocol is functional in terms of immunogenicity. Although the role of autoantibodies to melanogenesis MAA is not well understood in melanoma patients, they may have some effector role. Studies have shown that autoantibodies to intracellular proteins can be induced and can penetrate cells via Fc receptors and by other mechanisms (Alarcon-Segovia *et al*, 1996). It is becoming more evident that individual MAA proteins can elicit both T cell and antibody responses; however, of the major MAA studied it is still not known which MAA or type of immune response(s) (antibody or T cell) is clinically relevant for effective control of malignant melanoma. Future studies will involve assessment of both T and antibody responses to individual melanogenesis MAA. The immunogenicity of anti-melanogenesis MAA IgG responses provides another tool for the rapid assessment of immunogenicity of cancer vaccines (whole cell, membrane lysate, or subunit) in patients.

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