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Generation of polyclonal rabbit antisera to mouse melanoma associated antigens using gene gun immunization

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Abstract

Lymphocytes from patients with melanoma have been used to clone melanoma associated antigens which are, for the most part, nonmutated melanocyte tissue differentiation antigens. To establish a mouse model for the use of these 'self' antigens as targets for anti-tumor immune responses, we have employed the mouse homologues of the human melanoma antigens Tyrosinase, Tyrosinase Related Protein-1 (TRP-), gp100, and MART-1. We sought to generate antisera against these proteins for use in the construction of experimental recombinant and synthetic anti-cancer vaccines, and for use in biologic studies. Using genes cloned from the B16 mouse melanoma or from murine melanocytes, we immunized rabbits with plasmid DNAs coated onto microscopic gold beads that were then delivered using a hand-held, helium-driven 'gene gun'. This strategy enabled us to generate polyclonal rabbit sera containing antibodies that specifically recognized each antigen, as measured by immunostaining of vaccinia virus infected cells. The sera that we generated specifically for TRP-1, gp100, and MART-1 recognized extracts of the spontaneous murine melanoma, B16. The identities of the recognized proteins was confirmed by Western blot analysis. The titers and specificities of these antisera were determined using ELISA. Interestingly, serum samples generated against murine MART-1 and gp100 developed antibodies that were cross-reactive with the corresponding human homologues. Recognition of human gp100 and murine Tyrosinase appeared to be dependent upon conformational epitopes since specificity was lost upon denaturation of the antigens. These antisera may be useful in the detection, purification and characterization of the mouse homologues of recently cloned human tumor associated antigens and may enable the establishment of an animal model of the immune consequences of vaccination against 'self' antigens.

Keywords

Melanoma antigen; Recombinant anti-cancer vaccines; Gene gun immunization; Polyclonal antisera

1. Introduction

Recently, a number of melanoma associated antigens (MAA) which are recognized by tumor reactive T cells have been identified. These MAA are, for the most part, nonmutated melanocyte differentiation antigens and include: MART-1/MelanA, gp100, Tyrosinase, and Tyrosinase Related Protein-1 (TRP-1) (Robbins and Kawakami, 1996; Rosenberg, 1997; Sakai et al., 1997). Clinical trials are underway in patients with melanoma which use these MAA in anti-cancer vaccines. The targeting of these self antigens via immunotherapeutic strategies raises issues about the breaking of tolerance to normal self proteins as well as the autoimmune consequences of vaccination against target proteins found on both normal and cancerous cells (Overwijk et al., 1998). In patients responding to interleukin-2 (IL-2) therapy for melanoma,

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destruction of normal melanocytes resulting in vitiligo has been observed, but not in patients receiving IL-2 for renal cancer. Efforts are being made to establish a mouse model which mimics human autoreactivity responses to tumor antigens so these issues can be explored.

Mouse tumor models have often employed experimental tumors which are genetically altered to express a model tumor antigen usually of viral or bacterial origin (Wang et al., 1995; Pan et al., 1995). We are currently directing our efforts towards the use of the B16 melanoma from which the murine homologues of human melanoma associated antigens have been cloned (Zhai et al., 1997; Shibahara et al., 1986; Yamamoto et al., 1987). These mouse homologues are 'self' proteins which mirror the human situation more closely than a foreign antigen. It has been shown that cells specific for mgp100 can be expanded ex vivo and, upon adoptive transfer, can treat established B16 lung metastases (Overwijk et al., 1998). Further, mice immunized with rVVmTRP-1 are protected from subcutaneous and intravenous challenge with B16 (Overwijk et al., 1998). In these studies, mgp100 and mTRP-1 were classified as true tumor regression antigens.

To aid us in the development of a mouse model, we attempted to generate a source of antibodies that could specifically detect each of the mouse MAA. These antibodies were of potential usefulness in the development of recombinant and synthetic anti-cancer vaccines. They can be valuable reagents in the generation and characterization of recombinant virus-based vaccines (e.g., poxviruses, adenoviruses, influenza viruses) where they can be employed for immunostaining and plaque selection and for protein-based vaccines where they can be used for affinity chromatography and Western blotting. Additionally, these reagents may prove effective for the evaluation of tumor and tumor extracts by ELISA, flow cytometry, and the staining of frozen and paraffin embedded tissue sections. Such antibodies could also be useful in studies of the molecular and cell biology of these antigens as well as in their purification for immunologic and biochemical purposes.

Methods for making antisera include injection of proteins purified from tissue or cell lines, baculovirus generated proteins in adjuvant, and synthetic peptides coupled to KLH to increase immunogenicity. Often, the production and isolation of sufficient quantities of protein can be difficult and time consuming. The making of baculovirus can be especially laborious as one must first clone the gene of interest into a baculovirus vector, perform a recombination, select for recombinants, and produce a high titer viral stock. This is not always possible if a protein product is toxic in large amounts. Consideration must be given to whether the recombinant protein is secreted or cellular, and purification must be carried out accordingly. Levels of protein production can be variable and each step of infection and purification must be optimized for individual proteins.

More recently, genetic immunization has been used in order to circumvent the need for making purified protein (Tang et al., 1992; Barry et al., 1994; Krasemann et al., 1996). We and others have shown that humoral and cellular immune responses can be elicited by introducing genes via particle bombardment (Irvine et al., 1996). Here, we have used an easy method of eliciting antibody formation by direct delivery of plasmid DNA (pDNA) via 'gene gun' immunization. This entailed the use of a hand-held helium-driven device which propels DNA coated gold beads into the epidermis of a rabbit resulting in the de novo expression of the protein antigen in vivo leading to a specific immune response (Haynes et al., 1996; Pertmer et al., 1995). By using gene gun immunization with pDNAs encoding the genes for the murine homologues of human tumor antigens, we have generated polyclonal rabbit sera that specifically react with the mouse MAA which are being studied in our laboratory; mMART-1, mgp100, mTyrosinase and mTRP-1.

2. Materials and methods

2.1. Plasmid construction and purification

Plasmids encoding the cDNAs of the four murine tumor antigens; mgp100, mTyrosinase, mTRP-1, and mMART-1 were constructed for immunization. The plasmid containing the mMART-1 cDNA was constructed by TA cloning of an RT-PCR fragment obtained from melanocytes from the skin on normal C57BL/6 mice using murine MART-1 specific primers into the PCRIII vector under the control of the CMV promoter as described previously (Zhai et al., 1997). The mgp100 cDNA, also generated by RT-PCR from normal melanocytes and TA cloned (Zhai et al., 1997). was further subcloned as an Spe I/Xba I fragment into the Avr II site of WRG7077 under a CMV promoter (a gift from Geniva, Middleton). The TRP-1 containing plasmid, pMT4 (Shibahara et al., 1986) (A gift from Dr. S Shibahara, Friedrich Miescher Institut, Basel, Switzerland). was cut with Eco RV and Eco57 I to liberate the TRP-1 cDNA. The 3' overhangs were repaired using T4 polymerase, and the blunt fragment was cloned into the EcoRV site of pcDNA3 under the CMV promoter. The cDNA for mTyrosinase contained in pUC 118 (Yamamoto et al., 1987) (kind gift from Dr. H. Yamamoto, Tohoku University, Sendai, Japan) was liberated using EcoRI and subcloned into the EcoRI site of pcDNA3 under the CMV promoter. Circular plasmid DNA was prepared by alkaline lysis of overnight bacterial cultures followed by double cesium banding.

2.2. Immunization

DNA was prepared by coprecipitation with 2 μ m in diameter gold beads with the addition of 200 μ l of 2.5 M CaCl₂ during vortex mixing as described (Fuller and Haynes, 1994). DNA coated gold particles were delivered via 'gene gun' bombardment to the shaved abdomen of female New Zealand White rabbits using the Accell gene delivery system (kindly provided by Geniva, Middleton). Each rabbit received six shots at 1 μ g DNA per shot for a total of 6 μ g per rabbit. To ensure proper entry into the epidermal layer, rabbits received two shots of DNA-coated gold at three different pressures of helium (300, 400, and 500 psi). Three rabbits per group were immunized and then boosted at three week intervals with pDNA encoding one of the following murine tumor antigens: mgp100, mTRP-1, mTyrosinase, or mMART-1. Boosting was carried out using the same protocol as the original immunization. Preimmune sera were taken prior to any immunization. Rabbit sera were harvested approximately three weeks following each immunization, prior to the subsequent immunization for evaluation in ELISA, Western blotting and immunostaining assays. Gene gun immunization and sera collection were performed according to institutional guidelines for animal care and handling.

2.3. Immunostain of vaccinia virus infected cells

In order to examine whether the serum samples obtained could recognize and bind to the native form of both the mouse and human antigens, BSC-1 monkey kidney cell monolayers were infected for 48 h with vaccinia virus expressing either human or mouse gp100, TRP-1, Tyrosinase or MART-1. Construction of these viruses has been described elsewhere (Zhai et al., 1997). Infection was performed in 24 well plates at an MOI sufficient to produce isolated plaques. Following infection, cells were gently washed with PBS and fixed with a 1:1 solution of acetone/methanol for 5 min. Cell layers were washed with PBS 1 $\times$ and with 2% nonfat dried milk in PBS 1 $\times$. Rabbit sera were diluted 1:200 in 2% nonfat dry milk in PBS and 0.5 ml/well was incubated for 2 h at room temperature with gentle agitation. After washing the wells with 2% milk, the cell layers were incubated for 1 h with horseradish peroxidase-conjugated donkey anti-rabbit IgG F(ab')₂ fragments diluted 1:1000 (Amersham International, Amersham, UK). Bound immunoglobulin was then detected by incubating fixed cells for approximately 5 min in 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO) dissolved in dH₂O. The reaction was stopped by washing the wells with PBS.

2.4. Enzyme-linked immunosorbent assay

Serum from immunized rabbits was collected 3 weeks after each boost to analyze for the presence of murine and human tumor antigen-specific antibodies by ELISA using plate bound tumor lysate or vaccinia virus infected BSC-1 lysate. Specifically, BSC-1 cells were infected at an MOI of 5 with recombinant vaccinia virus expressing one of the following: hgp100, hTyrosinase, hMART-1, mTRP-1, mgp100, mTyrosinase, or mMART-1. Cells were incubated for approximately 48 h and then scraped and collected. Cells were washed in PBS and resuspended at 6×10^6 cells/ml in PBS. B16 melanoma cells were collected, washed, and resuspended at 1×10^7 cells/ml in PBS. Lysates were made by performing 3–4 cycles of freeze/thaw in liquid nitrogen and a 37°C water bath until no live cells were detected via trypan blue staining. BSC-1 cell lysates were then diluted 1:20 in DPBS, and 50 μ l/well (1.5×10^4 cell equivalents) were coated onto microtiter plates by drying overnight in a dry 37°C incubator. Tumor lysates were diluted at either 1:20 (for gp100 and TRP-1) or 1:4 (for tyrosinase and MART-1) and 50 μ l added to each well (2.5×10^4 or 1.25×10^5 cell equivalents). Plates were blocked with 2% nonfat dried milk in PBS in a humidified 37°C incubator for 1 h. Rabbit sera was diluted as indicated in 2% milk and 50 μ l were incubated for 1 h at 37°C. Plates were washed 5 $\times$ with 1% bovine serum albumin in PBS and incubated with a horseradish peroxidase donkey anti-rabbit IgG F(ab')₂ fragments diluted 1:2000 in milk for 1 h. The resulting complex was detected by chromogen, *o*-phenylenediamine (Sigma, St. Louis, MO). Absorbance at 492 nm was read on a Titertek Multiskan Plus reader (Flow Laboratories, MacLean, VA).

2.5. Western immunoblotting

Western blots were performed to ascertain whether serum samples could recognize protein bands of the predicted size. To this end, 10 μ l (6×10^4 cell equivalents) of virally infected BSC-1 cell lysate prepared as above was boiled in equal volume of SDS-PAGE sample buffer and resolved on a 8–16% gradient polyacrylamide gel (All protein electrophoresis and Western blotting reagents from Novex, San Diego, CA). These were electrotransferred onto nitrocellulose membranes (0.45 μ m pore size) for 2 h at room temperature in transfer buffer. The blots were blocked overnight at 4°C in PBS containing 5% nonfat dried milk. Ten ml of 1:200 dilution of antiserum in PBS containing 2% nonfat dry milk were added to each blot and incubated for 2 h at room temperature with gentle agitation. After washing the blots 3 $\times$ for 10 min in PBS containing 0.05% Tween-20, the blots were incubated for 1 h with horseradish peroxidase-conjugated donkey anti-rabbit IgG F(ab')₂ fragments (1:1000). Blots were washed extensively as above and bound immunoglobulin was detected by incubation for approximately 3 min in 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO). The reaction was stopped by washing for 5 min in H₂O.

2.6. Antibodies

Antibodies which were used as controls in these studies include the following: HMB45 (Vennegoor et al., 1988) (DAKO, Carpinteria, CA), M2-9 E3 (Kawakami et al., 1997) (supplied by Yutaka Kawakami, NCI). α -PEP 7 (Jiminez et al., 1991) (supplied by Vince Hearing, NCI), and T311 (Chen et al., 1995) (supplied by Lloyd Old, Sloan-Kettering Cancer Center).

3. Results

3.1. Gene gun immunization results in the production of anti-mouse MAA antibodies

DNA-mediated immunization against four different mouse MAA was performed on 3 rabbits per group. Rabbits were boosted every three weeks and sera was collected three weeks later, prior to the next shot of DNA. After the first boost, serum samples were tested for the presence of antibodies by their ability to specifically stain BSC-1 cells which had been previously

infected with a vaccinia virus expressing the appropriate mouse antigen (Fig. 1A). Control preimmune sera from the same rabbits was analyzed in parallel. Reactivity was seen after only one boost in all three of the rabbits which had been immunized with the pmgp100 vector as well as one rabbit in the pmTRP-1 group (Fig. 2). After subsequent boosts, antibodies were detected in additional samples. Post two boosts, an additional rabbit from the pmTRP-1 group became reactive as well as a rabbit from the pmMART-1 immunized group. The remaining rabbits from this group became reactive after the fourth boost. Additionally, antibodies were detectable in the serum of one of the pmTyrosinase immunized rabbits after the third boost, thus we were successful in obtaining positive sera from at least one animal per group and as many as all three. The remaining rabbits, two from the pmTyrosinase group and one from the pmTRP-1 group, did not develop reactivity following 5 boosts.

Serum samples which were positive for the presence of antibodies against the mouse MAA, were further tested against the human homologues of these genes. This was performed by the same procedure of staining virus infected cells using vaccinia virus expressing the human forms of the MAA, when available. The anti-murine Tyrosinase serum never showed a cross-reactivity with the human Tyrosinase protein within the time period tested. However, all samples from the pmgp100 group and one from the pmMART-1 group became cross-reactive after the second boost (Fig. 2). The remaining two anti-mouse . MART-1 sera did not show cross-reactivity, leaving us with both cross-reactive and non cross-reactive samples for these two antigens. A recombinant vaccinia virus expressing the human TRP-1 antigen was not available for us to test cross-reactivity.

3.2. Serum reactivity is specific for each MAA as shown by ELISA

To determine the degrees of reactivity and specificity, the sera of one positive rabbit from each group was used in an ELISA against vaccinia virus infected cell lysates. The sera obtained from the pmgp100 immunized rabbit after 3 boosts, demonstrated a titer of 1:6250 against rVVMgp100 infected BSC-1 cell lysate (Fig. 3C) However, the same sera only showed a titer of < 1:250 against lysed BSC-1 cells infected with either rVVMtyrosinase, rVVM MART-1 or rVVMTRP-1. Preimmunization sera failed to react with BSC-1 cells infected with rVVMgp100. Similarly, sera taken at the same time from the pm-MART-1 and pmTyrosinase immunized rabbits also showed specific activity against their respective antigens with titers of 1:1250 and 1:800, respectively, while prebleeds had titers of < 1:50 (Fig. 3D and 3A) While sera taken from the pmTRP-1 immunized rabbit after the third boost did not react specifically in this assay, a later serum sample from the same rabbit taken post 7 boosts recognized the rVVMTRP-1 lysate at a titer of approximately 1:1250 compared to preimmune sera which had a 1:50 titer (Fig. 3B). Not shown are previously described antibodies HMB45 which did not recognize either mouse or human gp100-expressing lysates, and M2-9 E3 which only recognized the human protein in this assay. These ELISA results demonstrated that rabbit sera were highly specific for the immunizing MAA.

3.3. Sera recognize proteins of the appropriate size on Western blot

The same sera used in the ELISA experiments were used in a Western blot against vaccinia virus infected BSC-1 cell lysates separated by SDS-PAGE. Using this method against both the mouse and human antigens, and staining with pmMART-1 immunized rabbit sera, bands were detected that match the previously reported sizes of approximately 18 kDa for the human protein (Kawakami et al., 1997) and 16 kDa for the mouse protein (Zhai et al., 1997) (Fig. 4B and C). An additional band which runs at approximately 36 kDa was also observed on these blots. While the identity of this band is unknown, it is likely that this band represents a homodimer of MART-1. A band corresponding to an estimated 85 kDa was recognized in the rVVMgp 100 infected cell lysate by the pmgp100 immunized rabbit serum but not the preimmune serum (not shown). Two previously described monoclonal antibodies, one against

human MART-1 (M2-9 E3) and one against gp100 (HMB45) did not stain these Western immunoblots. The human gp100 infected lysate were not recognized by this serum on Western blot, possibly due to the denaturing conditions since it stained whole cells infected with vaccinia virus containing the human gp100 gene (Fig. 1B). A definitive 75 kDa band was also detected by the pmTRP-1 immunized sera, but not by the control sera, in rVVmTRP-1 infected lysate (Fig. 4A). Since the human TRP-1 expressing vaccinia virus was not available, we did not test whether the antibodies cross-reacted with human TRP-1. Neither the human nor mouse Tyrosinase gene products were recognized by the rabbit serum on a Western blot, although the mouse Tyrosinase sera was positive by immunostain (Fig. 1C) and ELISA (Fig. 3A) implying recognition of an epitope that is conformationally dependant. Control antibodies, α -PEP7 and T311 confirmed the presence of mouse and human tyrosinase, respectively, in the lysates (not shown). Therefore, we have generated rabbit antisera that are able to recognize mouse, and in some cases human, melanoma antigens.

3.4. Serum reactivity to tumor lysate

To assess whether rabbit sera were able to recognize cells naturally expressing the target antigens, an ELISA was performed on B16 murine melanoma lysate. We tested the same sera that were previously used for an ELISA on vaccinia infected cell lysates shown in Fig. 3. The sera from the pmgp100 immunized rabbit as well as the pmTRP-1 immunized rabbit recognized B16 lysate at a titer of 1:5120 while preimmune sera from these rabbits had a titer of < 1:80 (Fig. 5B and C). MART-1 was recognized less well with a titer of 1:320 while preimmune serum titer was < 1:80 (Fig. 5A). Tyrosinase did not appear to be recognized in the B16 lysate (Fig. 5D). Low recognition by pmMART-1 and pmTyrosinase immunized rabbit sera appears to be due to low expression of the proteins by the tumor as both sera recognized BSC-1 cells infected with the proper antigen-expressing vaccinia virus (Fig. 3A and D), and a control anti-MART-1 antibody (M2-9 E3) and anti-Tyrosinase antibody (α -PEP 7) also failed to recognize B16 in this assay (not shown). A control tumor lysate, EL4, which does not express the MAA was not recognized by any of the sera (not shown).

4. Discussion

MART-1, gp100, TRP-1 and Tyrosinase are melanoma antigens that are recognized by tumor infiltrating lymphocytes (TILs) isolated from patients (Rosenberg, 1997). The clinical significance of these antigens is demonstrated by the fact that adoptive transfer of patient TILs can lead to tumor regression. We have shown that vaccination with these melanoma antigens in a mouse model can lead to the generation of an immune response. This has been done using recombinant viruses expressing these antigens and naked DNA administered intramuscularly or by gene gun [(Overwijk et al., 1998) and unpublished data]. The fact that all four of these antigens are nonmutated proteins which are found on normal melanocytes as well as on tumor raised important immunological questions such as: Would there be central or peripheral tolerance to these antigens? If so, would we be able to 'break' this tolerance in our attempts at eliciting anti-tumor immune responses? What would be the autoimmune consequences, if any, of breaking tolerance to normal 'self' proteins? Indeed, in our patients with metastatic melanoma, we occasionally see the development of vitiligo, a patchy and permanent loss of pigment in the skin and hair, especially in those patients who respond favorably to IL-2 (Rosenberg and White, 1996). A similar pattern of vitiligo was observed in our mouse studies in which we immunized mice with a recombinant vaccinia virus expressing TRP-1 (Overwijk et al., 1998).

Antisera against the mouse homologues of human tumor rejection antigens could be useful reagents in the characterization and monitoring of our vaccination strategies in this pre-clinical model. While antibodies against MAA have been described by others in human (Kawakami et

al., 1997;Chen et al., 1995;Gown et al., 1986;Vennegoor et al., 1988;Chen et al., 1996) and mouse (Kameyama et al., 1993;Thomson et al., 1985) we found that some of these antibodies, which were raised against human proteins, did not always cross-react with the mouse proteins or did not recognize the protein in its denatured form on Western blot. Additionally, many are not commercially available. Therefore, we set out to produce polyclonal antisera in rabbits against the mouse MAA, mMART-1, mgp100, mTRP-1, and mTyrosinase.

We obtained antisera against the mouse homologues of these tumor antigens employing the method of gene gun immunization. In contrast to the often difficult and slow-paced production of protein or peptides which must be optimized for each protein, the purification of large amounts of DNA is faster, cheaper, and less complicated. In fact, genetic immunization may be the only option for antibody production for proteins which have structures that are destroyed by purification. It has also been shown that gene gun immunization is a viable method for the production of monoclonal antibodies by making hybridomas from the splenocytes of gene gun immunized mice (Barry et al., 1994). Gene gun delivery of DNA has an advantage over intramuscular immunization in that it requires far less DNA, often only nanogram quantities (Pertmer et al., 1995). This method of immunization has been shown to generate a predominantly Th2 response with mostly IgG1 antibody production while i.m. immunization results in a predominantly Th1 response and mostly IgG2a antibody production (Feltquate et al., 1997).

The antisera we have developed against the mouse MAA specifically stain cells which are infected with vaccinia viruses expressing these proteins (Fig. 1), but not cells infected with a vaccinia virus expressing an irrelevant protein, nucleoprotein from influenza virus (data not shown). Methods for the production of antibodies which recognize mouse MAA have been described by others (Kobayashi et al., 1994;Kawakami et al., 1997;Jiminez et al., 1991). These methods involve immunization with peptide, which can result in the production of antibodies which recognize only a small portion of the molecule; or with protein, a method which may produce antibodies which recognize immunogen exclusively in its denatured form. Immunizing with DNA which results in de novo production of the entire protein with proper folding and post translational modifications can produce antisera against multiple epitopes that may be both conformational and nonconformational (Attanasio et al., 1997).

Interestingly, in some cases, boosting multiple times with the DNA encoding the murine antigens led to cross-reactivity to the human antigens. Only the pmTyrosinase immunization failed to induce cross-reactivity with the respective human protein while both pmMART-1 and pmgp100 immunization resulted in recognition of their human counterparts. The human MART-1 and gp100 proteins have been reported to be 68.8% and 70% homologous to the mouse proteins (Zhai et al., 1997). On the other hand, the human and mouse Tyrosinase proteins share 81% homology (Bouchard et al., 1989) implying a more similar protein between species. If true for rabbit as well as human, this high degree of homology could account for the relative difficulty in induction of anti-tyrosinase antibodies (i.e., a requirement for more boosts and fewer positive animals than the other groups) and for the lack of human cross-reactivity.

We successfully induced antibody production in at least 1 rabbit from each group, and as many as all three with as few as one boost. Some of the rabbits required four boosts before reactive sera was obtained, and several never became demonstrably positive in the time period tested (5 boosts) No information is available on the degree of homology between mouse and rabbit MAA since the genes from the rabbit have not been sequenced. Thus, the possibility exists that the difficulty of induction of rabbit antisera recognizing certain murine antigens is because of a high degree of similarity between the proteins. On the other hand, vector characteristics are a second variable which may account for these differences. It was interesting to us that all three of pmgp100 immunized rabbits started producing antibody after only one boost and resulted

in the highest titers since this was the only group that received the WRG7077 vector. This vector contains the intron A which has been shown to increase the transcription of mRNA leading to higher expression of the transgene (Chapman et al., 1991). The vectors used for the other groups, PCR III and pcDNA3, contain the same CMV promoter as WRG7077, but do not contain the intron A sequence.

The antisera which we have generated have proven to be valuable reagents in our laboratory. We have used them to characterize antigen expression on fixed tumor cells by immunostain and in tumor cell lysates by Western blot, as well as to fix complement in microcytotoxicity assays against human melanomas. These reagents have been useful in the characterization of recombinant MART-1 and gp100 protein products which will be utilized in the development of an ELISA to test for the presence of human anti-MAA antibodies in the sera of MART-1 and gp100 immunized patients in our ongoing clinical trials. Additional uses include quantitation of tumor antigen expression in comparison to normal tissue by ELISA and flow cytometry. Other laboratories have used these sera to test expression of new, recombinant viral vectors. In addition to using these antisera to detect protein, they are also employed to purify protein for in vitro and in vivo applications. These reagents may also be useful for studies of vitiligo or other studies of pigmentation.

5. Conclusion

In conclusion, the antisera we have elicited with plasmid DNAs encoding the murine MAA; TRP-1, Tyrosinase, gp100, and MART-1 appear to be effective reagents for the detection and characterization of these antigens. Clinical trials are currently underway in patients with metastatic melanoma to test the efficacy of tumor antigen targeted treatments in causing the regression of cancer. In order to address questions regarding safety as well as to study the optimization of treatment, and to assess the effect of autoreactivity to normal melanocyte specific proteins, we are designing anti-cancer vaccines based on the mouse homologues of gp100, MART-1, Tyrosinase and TRP-1 cloned from the model murine B16 melanoma.

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Abbreviations

ELISA	enzyme linked immunosorbent assay
MAA	melanoma associated antigen
MOI	multiplicity of infection
TRP-1	tyrosinase related protein-1
pDNA	plasmid DNA
rVV	recombinant vaccinia virus

Fig 1. Rabbit antisera specifically recognize BSC-1 cells infected with MAA-expressing vaccinia viruses

BSC-1 cell monolayers were infected with recombinant vaccinia viruses expressing mouse or human melanoma antigens, and stained with rabbit sera. Representative results are shown.

Mouse gp100 immunized rabbit serum stains rVVmgrp100 and rVVhgrp100 infected cells (A and B), but not rVVmtyrosinase or rVVhtyrosinase infected cells (D and E). Mouse tyrosinase immunized rabbit serum recognizes rVVmTyrosinase infected BSC-1 cells (F), but not rVVmgrp100 infected cells. (C). Similar results were obtained with MART-1 immunized sera which stains both mouse and human MART-1 vaccinia infected cells and with TRP-1 immunized rabbit sera which stains rVVmTRP-1 infected cells.

Rabbits Immunized With:

Fig 2. Repeated boosting leads to increased reactivity of sera

The complete results of the immunostaining data were compiled to make this figure. After one boost, all three gp100 immunized rabbits produced gp100 reactive sera. Additionally, rabbit C from the TRP-1 immunized group was positive at this time point. Following two boosts, rabbit C from the MART-1 immunized group as well as an additional TRP-1 immunized rabbit, rabbit B, had positive sera. Rabbit B from the tyrosinase immunized group and rabbits A and B from the MART-1 group became positive after 3 or 4 boosts, respectively. The remaining rabbits, A and C from the tyrosinase group, and A from the TRP-1 group did not develop reactivity in 5 boosts. When sera was examined for cross-reactivity to the corresponding human antigens, all three rabbits from the gp100 group and one, rabbit C, from the MART-1 group were cross-reactive after 2 boosts. The tyrosinase reactive serum and the remaining MART-1 reactive serum, A and B, were not cross-reactive after 5 boosts. TRP-1 reactive sera were not tested for cross-reactivity in this assay. NT = not tested, m = mouse reactivity, h = human reactivity.

Fig 3. Antisera specifically recognize MAA-expressing vaccinia virus infected BSC-1 cell lysates by ELISA

An ELISA was performed on vaccinia virus infected BSC-1 cell lysates using rabbit antisera at the indicated dilutions. The gp100 immunized rabbit serum specifically recognizes rVVm gp100 infected BSC-1 cell lysate with a titer of 1:6250 at OD₄₉₂ of 0.4 (C). Sera from tyrosinase and MART-1 immunized 492 rabbits showed specific activity against their respective antigens (A and D), with titers of 1:1250 and 1:800. Serum from the TRP-1 immunized rabbit recognizes rVVm TRP-1 infected cell lysate with a titer of 1:1250 at OD₄₉₂ of 0.6 (B).

Fig 4. Antisera specifically recognize appropriate bands in MAA-expressing BSC-1 cell lysates by Western blot

A Western blot was performed on vaccinia virus infected BSC-1 cell lysates and stained with rabbit antisera. Representative results are shown. The TRP-1 immunized rabbit serum, but not the preimmune control serum, detected a 75 kDa band in the rVVmTRP-1 infected cell lysate (A). Bands at approximately 18 kDa were recognized in both the rVVmMART-1 and rVVhMART-1 infected lysates by the MART-1 immunized rabbit serum, but not by the preimmune serum (B and C). Not shown is a Western blot of rVVmgp100 infected cell lysate in which an 85 kDa band was specifically recognized by the anti-gp100 antiserum, but not by preimmune serum.

Fig 5. Serum reactivity to B16 melanoma lysate

An ELISA was performed on B16 murine melanoma lysate using rabbit antisera at the dilutions indicated. Tumor lysate is specifically recognized by sera from rabbits immunized with mMART-1 (A), mgp100 (B) and mTRP-1 (C); but not by mTyrosinase immunized rabbit serum. (D) or by preimmune serum.