Immunogenicity of Human Melanoma-associated Antigens Defined by Murine Monoclonal Antibodies in Allogeneic and Xenogeneic Hosts

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ABSTRACT

The immunogenicity in patients with melanoma, in monkeys, and in rabbits of four human melanoma-associated antigens (MAA) defined by murine monoclonal antibodies was investigated. The latter included the high molecular weight MAA and the (M, 115,000, 100,000, and 95,000–150,000 MAA. To this end sera from patients with melanoma, from monkeys, and from rabbits immunized with cultured human melanoma cells were tested for their ability to inhibit the binding to cultured human melanoma cells of radiolabeled anti-M, 95,000–150,000 MAA monoclonal antibody (MoAb) 140.72, anti-high molecular weight MAA MoAb 225.28, anti-M, 115,000 MAA MoAb 345.134, and anti-M, 100,000 MAA MoAb 376.96. None of the sera from patients with melanoma significantly inhibited the reactivity of any of the anti-MAA monoclonal antibodies with melanoma cells. Of the sera from the six monkeys immunized with human melanoma cells, two sera significantly inhibited the reactivity with cultured human melanoma cells of both MoAb 345.134 and 376.96, one serum inhibited only that of MoAb 345.134, and the remaining three sera did not inhibit any of the four anti-human MAA monoclonal antibodies. Sera from six of the seven rabbits immunized with cultured human melanoma cells inhibited the binding to melanoma cells of at least one of the four anti-human MAA monoclonal antibodies while the serum from one rabbit immunized with a melanoma cell extract had no effect. Marked differences were found among the individual rabbit sera in their ability to inhibit the binding of the four anti-human MAA monoclonal antibodies. Sequential immunoprecipitation experiments corroborated the serological findings obtained with one of the two rabbit antisera tested. These results suggest that the immunogenicity of human MAA in mice may be different from that in patients with melanoma and in other animal species.

INTRODUCTION

Through the use of murine monoclonal antibodies a number of human MAA (i.e., antigens expressed by melanoma cells but not detectable in resting melanocytes) have been identified (for review, see Refs. 1 and 2). They have different structural profiles and display distinct distribution patterns in normal and malignant tissues. The relationship of most of these antigens to those identified in the past with sera from patients with melanoma and with sera from animals immunized with human melanoma cells or with MAA at various stages of purification (for review, see Refs. 3 and 4) is not known, since limited information is available about the structural properties of MAA defined by the latter type of reagents. Furthermore, the information about the immunogenicity of human MAA defined by murine monoclonal antibodies in patients with melanoma and in other animal species is scanty. Therefore, in the present investigation we have utilized both serological and immunoclinical approaches to determine whether patients with melanoma and various animal species immunized with human melanoma cells or soluble MAA have antibodies that recognize MAA defined by murine monoclonal antibodies.

MATERIALS AND METHODS

Cells. The cultured human melanoma cell lines Colo 38 and CaCL 78-1 were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mm l-glutamine, and 10 µg/ml gentamycin sulfate at 37°C in a humidified atmosphere containing 5% CO2.

Monoclonal Antibodies. The MoAb 149.53, 225.28, 657.5, and 763.74 to distinct determinants of the human HMW-MAA (5–7), the MoAb 345.134 to a M, 115,000 MAA (8), MoAb 376.96 to a M, 100,000 MAA (9), and the MoAb 140.72 (10) to a M, 95,000–150,000 MAA were developed and characterized as described. The MoAb 140.72 was metabolically labeled with [35S]methionine and purified from the culture medium by Protein A affinity chromatography (11). The remaining monoclonal antibodies were purified from ascites by caprylic acid precipitation (12) and radiolabeled with 125I by the chloramine-T method (13).

Sera from Patients with Melanoma. Sera from patients with melanoma and from apparently healthy individuals were sent under code to one of us (S. F.) by Dr. P. O. Livingston at Sloan Kettering Memorial Hospital (New York, NY) and by Dr. P. B. Dent, Department of Pediatrics, McMaster University (Hamilton, Ontario, Canada). The immunizing antigens which melanoma patients received and literature references (14–16, 43, 44) describing the patients’ sera are summarized in Table 1.

Conventional Xenoantisera. The sera AGMS-28, AGMS-56, and AGMS-61 were from monkeys immunized with cultured human melanoma cell lines CaCL 74-36, CaCL 78-1, and CaCL 78-4, respectively, as described by Liao et al. (17). Preimmune sera were obtained from each animal before the immunization was started. The sera A375, MeWo, and S-13 were from monkeys immunized with the corresponding human melanoma cell lines as described by Brüggen et al. (18).

Serum 3638 was from a rabbit primed with an i.v. injection of 1 × 106 cultured human melanoma cells Colo 55 and boosted with i.m. injections of 4 × 109 and 1 × 108 melanoma cells Colo 55 on Days 20 and 69, respectively. Bleedings were performed at weekly intervals following the first booster. Sera 7052 and 7053 were from rabbits immunized with monthly i.m. injections of 1 × 105 cultured melanoma cells Colo 55 which had been mixed with 300 µl of serum from a rabbit hyperimmunized with cultured human B lymphoid cells NC37. Bleedings were performed at weekly intervals following the first booster. Sera R55, R56, R57, and R88 were produced in rabbits given injections of the melanoma cell lines CaCL 73-36, CaCL 74-36, CaCL 78-1, and CaCL 78-4, respectively. Rabbits initially were given injections in 2 s.c. sites and 1 i.m. site with a total dose of 2.5 × 109 cultured human melanoma cells emulsified in 2 ml of Freund's complete adjuvant. At 2-wk intervals thereafter, rabbits were given bleedings in 2 s.c. sites with the same dose of melanoma cells suspended in 1.5 ml of Freund's incomplete adjuvant. Two wk after the fourth injection rabbits were exsanguinated. Preimmune sera were obtained from each animal 4 wk prior to the first immunization. Serum 9453 was from a rabbit immunized with antigens extracted with 3 M KCl from the cultured human melanoma cells.
melanoma cells M21 and fractionated on a KBr gradient by ultracentrifugation (19). Serum 2953 was from a rabbit immunized with the cultured human B-lymphoid cell line RPMI 6410.

Affinity purified xenon antibodies to murine Ig were purchased from Cooper Biomedical, Inc., Malvern, PA.

Serological Assays. The binding assay with 125I-labeled Protein A was performed as described (20). Briefly, Protein A was purchased from Pharmacia, Inc. (Piscataway, NJ) and labeled with 125I utilizing the chloramine-T method (13). Undiluted sera (50 µl) were incubated with 2 x 10^6 cells/well of cultured human melanoma cells Colo 38 for 1 h at 4°C. Cells were then washed 3 times with HBSS/BSA and incubated with 125I-labeled Protein A (1 x 10^5 cpm/well). After a 1-h incubation at 4°C, cells were washed 3 times with HBSS/BSA and bound radioactivity was measured with a gamma counter.

The inhibition binding assay with radiolabeled monoclonal antibodies was performed in 96-well round-bottomed polystyrene flat-bottomed plates (Becton Dickinson, Oxnard, CA) by incubating cultured melanoma cells (2 x 10^6 cells/well) with undiluted antisera (50 µl/well) for 1 h at room temperature. Then cells were washed 3 times and incubated for 1 h at room temperature with 125I-labeled monoclonal antibodies (1 x 10^5 cpm/well). After the incubation period, cells were washed 3 times with HBSS/BSA and bound radioactivity was measured with a gamma counter. Negative controls were performed by incubating melanoma cells with normal sera. Positive controls were performed by incubating melanoma cells with unlabeled monoclonal antibody homologous to the radiolabeled monoclonal antibody. The data were expressed as the percentage of inhibition of radiolabeled monoclonal antibody binding to melanoma cells.

Cell Surface Radialabelling with Lactoperoxidase. Cultured melanoma cells were surface labeled with 125I by a modification of the lactoperoxidase method published by Goding (21). Briefly, a minimum of 1 x 10^7 cells harvested by treatment with 1 mM EDTA in PBS (10 mM phosphate, 150 mM NaCl, pH 7.2) were washed twice with PBS, suspended in 250 µl of PBS containing 50 µg of lactoperoxidase, and mixed with 500 µCi of 125I. The iodination reaction was catalyzed by the sequential addition at 3-min intervals of 10-µl aliquots of 1:9000, 1:3000, and 1:1000-dilutions of 30% hydrogen peroxide in PBS. After the final incubation cells were washed with PBS and lysed in PBS buffer containing 1% NP-40, 1 mM EDTA, and 0.1% BSA. Nuclei were removed by centrifugation at 400 x g for 5 min and the supernatant lysate was clarified by centrifugation at 10,000 x g for 1 h. Prior to immunoprecipitation or immunodepletion experiments cell lysates were preclayed by a 1-h incubation with PAS beads (Pharmacia, Inc., Piscataway, NJ) coated with rabbit anti-mouse Ig antibodies.

Indirect Immunoprecipitation, Sequential Immunodepletion, and SDS-PAGE. NP-40 lysates of 125I-labeled surface melanoma cells (2 x 10^6 cpm) were incubated overnight with 250 µl of hybridoma supernatant. Antigen/antibody complexes were precipitated by overnight incubation at 4°C with 10 µl of PAS beads previously coated with an excess of rabbit anti-mouse Ig antibodies. Beads were washed sequentially with lysing buffer containing 0.5 M NaCl, with lysing buffer containing 0.1% SDS, and with PBS. Immunoprecipitated antigens were released from PAS beads by boiling in 100 µl of SDS sample buffer and loaded on 5-12% acrylamide gradient slab gels. Following SDS-PAGE radioactive bands were detected by exposing dried gels to X-ray film (XAR-5, Eastman Kodak, Rochester, NY) at −80°C for periods of time ranging from 2 days to 2 wk. Molecular weight determinations were made by comparing the position of immunoprecipitated components with the migration of 125I-labeled molecular weight markers (Bio-Rad Laboratories, Richmond, CA) included as standards in each gel.

Immunodepletion analyses were performed by incubating NP-40 lysates of 125I-labeled melanoma cells with PAS beads coated with Ig from rabbit sera. Following three sequential incubations at 4°C for 1 h with antisera, lysates were divided and immunoprecipitated with murine monoclonal antibodies. Antigens precipitated by monoclonal antibodies from lysates depleted with antisera were analyzed by SDS-PAGE and compared to lysates depleted with control sera for the loss or reduction of immunoprecipitated MAA.

Statistical Analysis. Results from 125I-labeled Protein A binding assay were assessed for statistical significance by applying the one-tailed t test to data transformed by the relation: X = square root of cpm bound. Sera with binding levels greater than the 95% confidence limits calculated for the mean binding values of normal and/or preimmune sera were classified as positive for reactivity to cultured melanoma cells. Data from competition binding assays were transformed by the relation: X = arcsin of the square root of the percentage of inhibition and the results were assessed for statistical significance by application of the t test as described above.

RESULTS

Sera from 10 patients with melanoma, 6 monkeys, and 7 rabbits that had been immunized with human melanoma cells were incubated with the cultured human melanoma cell lines Colo 38 and CaCl 78-1. Following three washings, cells were assayed for the binding of 125I-labeled anti-HMW-MAA MoAb 225.28, 125I-labeled anti-M, 115,000 MAA MoAb 345.134, 125I-labeled anti-M, 100,000 MAA MoAb 376.96, and 35S-labeled anti-M, 95,000–150,000 MAA MoAb 140.72 and the percentage of binding inhibition was calculated.

As measured by the 125I-labeled Protein A binding assay the reactivities to cultured human melanoma cells of postimmunization sera SK-3, SK-5, SK-7, PD-1, PD-3, and PD-4 from patients with melanoma were significantly greater (P < 0.05 for a one-tailed t test) than the reactivities of sera from apparently healthy individuals (SK-6 and SK-8) and sera from unimmunized patients with melanoma (SK-2, PD-5, and PD-6). However, none of the human sera significantly inhibited the binding of any of the four murine antihuman MAA monoclonal antibodies (Table 1). It is noteworthy that immunization of Patient 8 with allogeneic cultured melanoma cells did not result in any detectable increase in the ability of the postimmune serum (SK-3) to inhibit the binding of radiolabeled monoclonal antibodies to cultured melanoma cells when compared to the preimmune serum (SK-2). To examine the possibility that the sera from patients with melanoma contain antibodies which recognize determinants of the HMW-MAA different from that defined by MoAb 225.28, the sera were tested for their ability to inhibit the binding to melanoma cells of 125I-labeled MoAb 149.53, 657.5, and 763.74 to distinct determinants of the HMW-MAA. In no case did patients' sera inhibit the binding of the latter antibodies.

None of the sera from the 6 monkeys immunized with cultured human melanoma cells affected the binding of MoAb 140.72 and 225.28 to cultured human melanoma cells (Table 2). The antisera A-375, MeWo, and S-13 all inhibited the binding of MoAb 345.134 to melanoma cells with the levels of inhibition ranging from 55 to 78%. In addition, the antisera MeWo and S-13 inhibited the binding of MoAb 376.96 to melanoma cells by 87 and 57%, respectively.

The sera from the 7 rabbits immunized with cultured human melanoma cells and from the rabbit immunized with the melanoma cell extract differed markedly in their ability to inhibit the binding of anti-human MAA monoclonal antibodies to melanoma cells (Table 3). Antisera 9453 and RS6 did not inhibit the binding of any of the four anti-MAA monoclonal antibodies. Antiserum RS8 inhibited the binding of MoAb 140.72, 225.28, and 376.96. Antisera 3638 and 7053 inhibited the binding of MoAb 225.28, 345.134, and 376.96. Antisera 7052 and RS5 inhibited the binding of MoAb 345.134 and the antisera RS7 inhibited the binding of MoAb 376.96. There were no marked differences among the sera with inhibitory activity in the extent of blocking of the binding of MoAb 225.28 to melanoma cells. On the other hand, Antisera 3638 and 7053 had a much higher inhibitory activity than Antisera 7052 and...
IMMUNOGENICITY OF HUMAN MAA

Table 1 Inhibition of murine anti-MAA monoclonal antibody binding to human melanoma cells by sera from patients with melanoma

Inhibition values were not significantly greater than the inhibition by control sera at the 95% level of confidence for a one-tailed t test.

<table>
<thead>
<tr>
<th>Source</th>
<th>Code</th>
<th>Immunogen</th>
<th>Radiolabeled anti-MAA MoAb</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. O. Livingston*</td>
<td>SK-1</td>
<td>Autologous and allogeneic melanoma cells</td>
<td>140.72 225.28 345.134 376.96</td>
</tr>
<tr>
<td></td>
<td>SK-2</td>
<td>Autologous melanoma cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SK-3</td>
<td>Autologous melanoma cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SK-4</td>
<td>Autologous melanoma cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SK-5</td>
<td>Autologous melanoma cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SK-6</td>
<td>Autologous and allogeneic melanoma cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SK-7</td>
<td>Autologous and allogeneic melanoma cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SK-8</td>
<td>Autologous melanoma cells</td>
<td></td>
</tr>
<tr>
<td>P. B. Dent*</td>
<td>PD-1</td>
<td>Allogeneic melanoma cells + BCG</td>
<td>&lt;15  1  0  13</td>
</tr>
<tr>
<td></td>
<td>PD-2</td>
<td>Allogeneic melanoma cells + BCG</td>
<td>&lt;15  5  0  13</td>
</tr>
<tr>
<td></td>
<td>PD-3</td>
<td>From patient PD-2 2 yr after the first immunization</td>
<td>&lt;15 0 0 0</td>
</tr>
<tr>
<td></td>
<td>PD-4</td>
<td>Allogeneic melanoma cells + BCG</td>
<td>&lt;15 15 6  0</td>
</tr>
<tr>
<td></td>
<td>PD-5</td>
<td>Allogeneic melanoma cells + BCG</td>
<td>&lt;15 15 6  0</td>
</tr>
<tr>
<td></td>
<td>PD-6</td>
<td>Allogeneic melanoma cells + BCG</td>
<td>&lt;15 15 6  0</td>
</tr>
</tbody>
</table>

* SK-2 and SK-3 are pre- and postimmunization sera, respectively, from Patient 8 (14), SK-5 is serum from Patient CZ (15), and SK-7 is serum from patient BG (15, 16). SK-6 and SK-8 are sera from apparently healthy individuals.

* Percentage of inhibition of the binding of radiolabeled anti-MAA monoclonal antibody to cultured human melanoma cells.

Table 2 Inhibition of murine anti-MAA monoclonal antibody binding to human melanoma cells by sera from monkeys immunized with cultured human melanoma cells

Inhibition values were not significantly greater than the inhibition by pre-immune sera at the 95% level of confidence for a one-tailed t test.

<table>
<thead>
<tr>
<th>Monkey antisera</th>
<th>Immunizing melanoma cell line</th>
<th>Radiolabeled anti-MAA MoAb</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-375</td>
<td>A-375</td>
<td>NT* 10 55 21</td>
</tr>
<tr>
<td>AGMS-28</td>
<td>CaCL 74-36</td>
<td>20 17 21 0</td>
</tr>
<tr>
<td>AGMS-56</td>
<td>CaCL 78-1</td>
<td>12 7 29 21</td>
</tr>
<tr>
<td>AGMS-61</td>
<td>CaCL 78-4</td>
<td>13 18 48 20</td>
</tr>
<tr>
<td>MeWo</td>
<td>MeWo</td>
<td>NT 19 78 77</td>
</tr>
<tr>
<td>S-13</td>
<td>S-13</td>
<td>NT 0 60 57</td>
</tr>
</tbody>
</table>

* Not tested.

* Percentage of inhibition of the binding of radiolabeled anti-MAA monoclonal antibody to cultured human melanoma cells.

Table 3 Inhibition of murine anti-MAA monoclonal antibody binding to human melanoma cells by sera from rabbits immunized with cultured human melanoma cells

Inhibition values were not significantly greater than the inhibition by pre-immune sera at the 95% level of confidence for a one-tailed t test.

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Immunogen</th>
<th>Radiolabeled anti-MAA MoAb</th>
</tr>
</thead>
<tbody>
<tr>
<td>2953</td>
<td>B lymphoid cells RPMI 6410</td>
<td>0* 4 33 0</td>
</tr>
<tr>
<td>3638</td>
<td>Melanoma cells Colo 55</td>
<td>34 61 94 89</td>
</tr>
<tr>
<td>7052</td>
<td>Melanoma cells Colo 55</td>
<td>12 22 48 3</td>
</tr>
<tr>
<td>7053</td>
<td>Melanoma cells Colo 55</td>
<td>20 49 88 81</td>
</tr>
<tr>
<td>9453</td>
<td>Extract of melanoma cells M21</td>
<td>0 18 8 14</td>
</tr>
<tr>
<td>RS5</td>
<td>Melanoma cells CaCL 73-36</td>
<td>6 0 44 1</td>
</tr>
<tr>
<td>RS6</td>
<td>Melanoma cells CaCL 74-36</td>
<td>10 7 35 21</td>
</tr>
<tr>
<td>RS7</td>
<td>Melanoma cells CaCL 78-1</td>
<td>13 12 20 44</td>
</tr>
<tr>
<td>RS8</td>
<td>Melanoma cells CaCL 78-4</td>
<td>66 52 27 77</td>
</tr>
</tbody>
</table>

* Percentage of inhibition of the binding of radiolabeled anti-MAA monoclonal antibody to cultured human melanoma cells.

RS5 on the binding of MoAb 345.134 to melanoma cells. The blocking of the binding of MoAb 376.96 to melanoma cells by serum RS7 was markedly lower than that by Antiserum 3638, 7053, and RS8.

The specificity of the blocking in these assays is supported by the observation that none of the preimmune sera exceeded a 28% level of inhibition. Furthermore, Antiserum 2953 from a rabbit immunized with the cultured human B-lymphoid cell line RPMI 6410 did not inhibit the binding of any of the anti-MAA monoclonal antibodies to melanoma cells.

Sequential immunoprecipitation experiments were performed to define the molecular basis of the serological findings. Antiserum 3638 and 7053 which had the highest levels of blocking activity were tested for their ability to immunodeplete NP-40 extracts of cultured human melanoma cells of the molecules carrying the determinants recognized by the anti-MAA MoAb 225.28, 345.134, and 376.96. Antiserum 3638 removed the molecules recognized by MoAb 345.143 and 376.96 and significantly reduced the amount of antigen recognized by MoAb 225.28 (Fig. 1, middle). On the other hand, Antiserum 7053 which displayed a blocking pattern similar to that of Antiserum 3638 did not deplete any of the antigens recognized by MoAb 225.28, 376.96, and 345.134 (Fig. 1, right).

DISCUSSION

Serological and immunochemical studies have shown marked differences in the immune response of various animal species and of individual members within each species to the human HMW-MAA and to M, 115,000, 100,000, and 95,000-150,000 MAA defined by murine monoclonal antibodies. All four MAA were immunogenic in rabbits; the M, 115,000 and 100,000 MAA were immunogenic in monkeys. Antibodies able to inhibit the binding of the four anti-MAA monoclonal antibodies to melanoma cells were not detected in the sera of patients with melanoma.

It is noteworthy that the inhibition of the binding of MoAb 225.28 to melanoma cells and the immunodepletion of the HMW-MAA by rabbit Antiserum 3638 are only partial. In view of the heterogeneity in the expression of antigenic determinants on HMW-MAA-bearing molecules synthesized by cultured melanoma cells (6, 7, 22, 23), our serological and immunochemical findings suggest that the rabbit Antiserum 3638 and the MoAb 225.28 recognize distinct but spatially close antigenic determinants, which have a differential distribution on the pool of HMW-MAA-bearing molecules synthesized by cultured human melanoma cells Colo 38. On the other hand, the inhibition...
Depleted with: Normal Rabbit Serum

3638 7053

280 Kd —
100 Kd —
85 Kd —
30 Kd —

Precipitated with:

Control 225.28 345.134 376.96

Control 225.28 345.134 376.96

Control 225.28 345.134 376.96

Control 225.28 345.134 376.96

Fig. 1. SDS-PAGE analysis under reducing conditions of components immunoprecipitated by anti-HMW-MAA MoAb 225.28, anti-M, 115,000 MAA MoAb 345.134, and anti-M, 100,000 MAA MoAb 376.96 from 125I-labeled melanoma cell lysates which had been previously depleted with normal rabbit serum (left), rabbit Antiserum 3638 (middle), and rabbit Antiserum 7053 (right). A lysate of 1 x 10^6 125I-labeled cultured human melanoma cells Colo 38 was incubated 3 times at 4°C for 1 h with PAS beads coated with immunoglobulins from the rabbit sera. Each immunodepleted lysate was divided into 4 aliquots and precipitated with a mouse myeloma protein as a negative control and with MoAb 225.28, 345.134, and 376.96. It should be noted that the M, 115,000 MAA comprises a M, 85,000 and 30,000 subunit which are linked by sulfydryl bonds. Kd, molecular weight in thousands.

of the binding of MoAb 345.134 and 376.96 to melanoma cells and the immunodepletion of the M, 115,000 and 100,000 MAA by rabbit Antiserum 3638 are complete. These results suggest that the latter reagents and the monoclonal antibodies recognize the same or spatially close antigenic determinants which are expressed on the same populations of MAA-bearing molecules. Although it inhibited the binding of the anti-MAA monoclonal antibodies to an extent similar to that of Antiserum 3638, the Antiserum 7053 did not immunodeplete the melanoma cell extract of the molecules recognized by the anti-MAA monoclonal antibodies. These results suggest that the rabbit antibodies either react with molecules distinct but close to those recognized by monoclonal antibodies or have too low an affinity to deplete MAA from a melanoma cell extract.

Limited information is available in the literature about the immunogenicity of MAA defined by murine monoclonal antibodies in patients with melanoma and in other animal species. The monosialoganglioside GM2 and the disialogangliosides GD2 and GD3 which are expressed abundantly by most malignant melanomas have been shown to be immunogenic in mice and patients with melanoma (24–35). Bystryn et al. (36) analyzed sera from two rabbits immunized with partially purified antigens shed by cultured human melanoma cells. The structural profile of antigens immunoprecipitated from cultured human melanoma cells by the two rabbit antisera were different from those of antigens immunoprecipitated by a panel of murine anti-human MAA monoclonal antibodies, including MoAb 225.28, 345.134, and 376.96. The discrepancy between the results of Bystryn et al. (36) and our own may reflect the different types of immunogens used in the two investigations, especially in view of the fact that the MAA recognized by MoAb 225.28, 345.134, and 376.96 are shed in limited amounts by melanoma cells (37). Alternatively the conflicting results may reflect the individual variability in the immune response of rabbits to human MAA; if the latter is the case, the number of rabbits tested by Bystryn et al. (36) was too small to draw a general conclusion with confidence. At variance with our own results Seigler found antibodies to HMW-MAA in sera from chimpanzees and from patients with melanoma immunized with purified HMW-MAA. This discrepancy may reflect differences in the sensitivity of the assay system used to detect antibodies and/or in the immunogenicity of the immunogens used in the two investigations. Since we utilized cultured human melanoma cells, immunodominant antigens may have abrogated the immune response to HMW-MAA. Alternatively the amount of HMW-MAA given by injection might not have been sufficient to elicit a humoral immune response.

Several mechanisms can be postulated to account for our inability to detect antibodies in the sera of patients with melanoma which react with the determinants defined by murine anti-MAA monoclonal antibodies. Immunization did not occur since the patients' melanoma lesions and the cultured melanoma cells used as immunogens did not express the antigenic determinants recognized by the monoclonal antibodies tested. This possibility is unlikely since the MAA identified by the monoclonal antibodies tested have been found in a high percentage of melanoma lesions and melanoma cell lines (38). Furthermore the MoAb 225.28, 345.134, and 376.96 react with the melanoma cell lines SK-Mel-37 and SK-Mel-93 which were used as immunogens in patients SK-3 and SK-4. Alternatively
the MAA defined by murine monoclonal antibodies are not immunogenic in man. This possibility is also unlikely in view of the immunogenicity of human MAA in animal species which are phylogenetically close to man. Another possibility is that the patients' antibody repertoire is different from that of other animal species and that the immune response is directed at determinants on MAA distinct and spatially distant from those recognized by murine monoclonal antibodies. In this regard, only some of the anti-HMW-MAA monoclonal antibodies tested have been reported to inhibit the cytoxicity of T-cell clones toward autologous melanoma cells (39, 40). Abnormalities of the patients' immune systems associated with malignant disease could prevent a humoral response to MAA. Although the sera tested in this study were from patients who were responsive to recall antigens at the time of immunization and who often developed elevated antibody titers to fetal calf serum components and alloantigens expressed on the immunizing cells (41), this mechanism cannot be excluded since specific immune suppression to MAA could occur in the patients investigated. The antibodies could complex with shed antigens and/or anti-idiotypic antibodies although immune complexes are not frequently detected in sera from patients with melanoma (42). On the other hand antibodies present in patients' sera may have lower affinity than murine anti-MAA monoclonal antibodies and therefore become displaced in the blocking assay.

From a methodological viewpoint the serological and immunochemical procedures we have applied are worth being commented upon: inhibition binding assays with radiolabeled monoclonal antibodies avoid time-consuming absorption procedures and allow the detection of antibodies present in small amounts, even if mixed with complex populations of antibodies of various specificities. The procedure appears to be specific since incubation of melanoma cells with sera elicited by immunization with lymphoid cells does not affect the reactivity with anti-MAA monoclonal antibodies. However, two limitations of this procedure should be stressed: false-negative results may occur if the affinity of the antibodies present in sera is lower than that of the monoclonal antibodies being tested. On the other hand, false-positive results may occur because of blocking by steric hindrance of antibodies reacting with molecules spatially close but distinct from those recognized by the monoclonal antibodies being tested. The latter limitation can be overcome by combining the inhibition binding assay with sequential immunoprecipitation experiments, provided that the affinity of the antibodies in the antiserum is high enough to immunoprecipitate the molecules recognized by the monoclonal antibodies being tested.

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