Monoclonal Anti-Idiotypic Antibodies to Human Melanoma-associated Proteoglycan Antigen: Generation and Characterization of Anti-Idiotypic Antibodies

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ABSTRACT

We have characterized a number of monoclonal anti-idiotype antibodies (mAb2s) made against a monoclonal antitumor antibody, MEM136. The monoclonal antibody 1 (mAb1) MEM136 recognizes an epitope on human melanoma-associated proteoglycan and blocks melanoma cell interaction with basement membrane components in vitro. The anti-idiotype antibodies (Ab2s) made against MEM136 each cross-inhibited, to varying degrees, their binding to MEM136. Thus, the mAb2s recognized overlapping idiotopes on MEM136. In an attempt to identify potential internal image candidates we set up a cell migration inhibition assay. In this assay, migration of melanoma-associated proteoglycan-positive Colo38 cells was determined through a membrane barrier impregnated with Matrigel, which is composed of extracellular matrix components, i.e., collagen type IV, heparan sulfate, and laminin. Interestingly, only Ab2s IM06 and IM32 inhibited melanoma cell migration. Additional studies indicate that of eight mAb2s tested, only IM32 and IM06 induced anti-MPG responses in rabbits. The possibility that IM32 and IM06 bear images of melanoma cell surface-associated proteoglycan epitopes is discussed.

INTRODUCTION

MPGs

are expressed on the cell surface of more than 80% of melanomas and in lesser amounts on a variety of normal tissues including keratinocytes and endothelial cells (1-8). MPG molecules are believed to be involved in a variety of cellular events related to the biology of tumor growth, e.g., proliferation, migration, and adhesion (9-15). It has also been proposed that MPG may influence tumor growth by acting as receptors for components of the ECM and various circulating molecules (16). Proteoglycans are known to bind various components of the ECM, including fibronectin (17-19), laminin (20, 21), collagen (22, 23), and other proteoglycans (24). Therefore, it is likely that tumor growth could be impaired if MPG function is inhibited. This notion was supported by the demonstration that mAb2s to proteoglycan antigens have an inhibitory effect on early events of cell spreading on a basement membrane synthesized by endothelial cells (25).

Passive immunotherapy with mouse monoclonal antitumor antibodies could be beneficial in patients with tumors. Their effectiveness, however, is compromised by the fact that: (a) mouse antibodies have short half-lives in humans, thus requiring frequent infusions to maintain a desired level of antibody in circulation; (b) the induction of anti-idiotype and anti-constant region antibodies against mouse antibodies in humans interferes with effective targeting by passively infused antibodies; and (c) high doses of Ab1 are required to attain substantial localization at the affected sites (26-30). On the other hand, monoclonal anti-idiotope antibodies mimicking the MPG antigen can be used in active immunotherapy to induce long-lasting human anti-MPG humoral responses (anti-anti-idiotype or Ab1 response) in melanoma patients. Moreover, the presence of human antibodies directed against the constant region of a mouse Ab2 will not compromise effector functions mediated by Ab1.

MEM136 is a mAb which recognizes an epitope of undefined structure on the MPG molecule. When tested in an immunohistology study, MEM136 reacted strongly to 80% of melanoma tissues and minimally to normal tissues. In an effort to generate monoclonal anti-idiotope antibodies which functionally mimic an epitope detected by MEM136, we first characterized the specificity, biochemical, and biological properties of MEM136 and then generated and characterized several site-specific anti-idiotope antibodies. The V gene usage of these Ab2s was then analyzed by biochemical and immunochemical means, and a preliminary functional characterization of these antibodies was then performed in a melanoma cell migration assay to identify mAb2s mimicking epitopes on MPG antigen.

MATERIALS AND METHODS

Cell Lines. The human melanoma cell lines Colo38 and Meljur and the neuroblastoma cell line NMB7 were grown in RPMI 1640 supplemented with 10% calf serum, 1% glutamine, and 10 μg/ml gentamicin sulfate.

Monoclonal Antibodies. The anti-MPG mAb MEM136 (γ1, κ; Hybritech, San Diego, CA) was purified on a protein A-Sepharose 4B column. MOPC21 (γ1, κ; Bionetics, Charleston, SC) was used as an isotype-matched control for MEM136. The generation and characterization of anti-MPG mAb1s 225.28S, 149.53, 763.74T, and T41.2 have been described earlier (31, 32). The specificity of anti-MPG mAb 155.8 has also been reported (33). The Colo38 reactive anti-HLA class I mAb TP25.99 (γ1, κ) used as a control was kindly provided by Dr. S. Ferrone. An unrelated monoclonal anti-idiotype antibody, 1CS (γ1, κ), derived from A/J mice, was used as a control in some experiments. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG and goat anti-mouse IgG antibodies were purchased from Becton Dickinson (Mountain View, CA).

Immunohistology Study with MEM136. Primary and metastatic melanomas, a variety of tumors, and pigmented skin lesions which included nevi were tested for mAb MEM136 reactivity. Tumors and histologically normal adult human tissues were obtained from surgical pathology and autopsy specimens. Fresh tissues were embedded in Tissue Tek O.C.T. compound in cryomolds (Miles Laboratories, Inc., Naperville, IL), frozen, and stored at −70°C until needed. All tissues were used as frozen tissue sections obtained from a frozen tissue bank and were well preserved histologically. Immunohistology was performed as described earlier (34).
Preparation of F(ab')2 Fragments. Pepsin at a final concentration of 25 μg/ml was added to MEM136 (1–2 mg/ml in 0.1 M citrate buffer, pH 3.5), and the solution was incubated for 8 h at 37°C. The reaction was stopped by increasing the pH to 8.0 by adding high-salt protein A (pH 3.5), and the solution was incubated for 8 h at 37°C. The reaction formed as described by Newman (35). Briefly, Colo38 cells (1.5 x 10⁷) were cultured for 3 h in 1 ml methionine-free RPMI 1640 supplemented with 200 μCi [35S]methionine (Amersham International, Amersham, Bucks, England). At 1 h after incubation, the gels were destained with 10% acetic acid and 25% ethanol and then stained with the same solution containing 0.1% Coomassie blue; pi 3-10 isoelectric focusing gels (Novex, Encinitas, CA). After fixing, gels were dried and analyzed by autoradiography. The antibodies were produced as ascites fluids in BALB/c mice.

Selection of Anti-MEM 136 Idiotype Hybridomas. Antibody screening was performed by indirect immunoprecipitation from the hybridomas as described by ELISA using Fab' fragments of MEM136 and MOPC21 as described below. Cells containing antibody that bound to the MEM136 Fab' fragment were collected and subject to 6% polyacrylamide gel electrophoresis under reducing conditions (36). Gels were then exposed for autoradiography.

Isoelectric Focusing of Ab2 Antibodies. Antibody screening was performed by isoelectric focusing using pH 3–10 isoelectric focusing gels (Novex, Encinitas, CA). After fixing, the gels were destained with 10% acetic acid and 25% ethanol and then stained with the same solution containing 0.1% Coomassie blue; pi standards were included to determine pH values (Pharmacia LKB Biotechnology, Piscataway, NJ).

Idiotope Localization of Ab2 Antibodies. Immunoblotting assays were performed to determine whether the epitopes recognized by the anti-idiotype antibodies were detected on isolated heavy or light chains of MEM136. Monoclonal antibody MEM136 was reduced with 5% 2-mercaptoethanol. Its heavy and light chains were separated in a 10% polyacrylamide–sodium dodecyl sulfate gel and transferred onto nitrocellulose using a Transphor blotting apparatus ( Hoeffer, San Francisco, CA). As a positive control, unreduced MEM136 was run on the same gel. Control nitrocellulose strips were stained with Pronase S stain (Sigma Chemical Co., St. Louis, MO). Blots to be reacted with monoclonal anti-idiotype antibodies were first blocked for 2 h with BSA (3% in Tris-buffered saline). These blots were then incubated for 2 h with 125I-labeled monoclonal anti-idiotype antibodies (1 x 10⁶ cpm/ml in blocking buffer). After six washings with Tris-buffered saline, the blots were dried and analyzed by autoradiography. The antibodies were labeled by the chloramine-T method.

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The binding site specificity of the Ab2s for 18 h at 4°C. Thereafter, plates were washed, and bound antibody was measured in a gamma counter.

ELISA Assay. Fifty μl of 1 μg/ml of antibody was coated in 96-well polystyrene plate for 2 h at room temperature. Plates were blocked with 1% BSA in PBS for 1 h and washed in PBS, and different dilutions of supernatant or a known concentration of antibody was added. Plates were then incubated for 2 h and washed, and goat anti-mouse IgG conjugated to peroxidase was added. After a 1-h incubation, the plates were washed, and the peroxidase substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) was added. The color was developed, and the absorbance was determined at 405 nm.

Immunization of Rabbits. Adult New Zealand White rabbits (two per group) were immunized s.c. at different sites with 500 μg rabbit of IM04 through IM32 in muramyl dipeptide-derived adjuvant containing 250 μg of threonyl muramyl dipeptide (38). Control rabbits were immunized with 500 μg of MOPC21 in muramyl dipeptide-derived adjuvant. The results presented in this paper were obtained with sera taken 7 days after the second immunization.

Analysis of Ab3 Response. The idiotypic responses induced by IM04, IM06, IM08, IM09, IM13, IM20, and IM32 in rabbits were analyzed by RIA as described earlier (37). The results were expressed as percentages of inhibition of 125I-A2 binding to MEM136, as compared to the binding performed in the presence of preimmune sera.

Serological Assays. IIF was performed by incubating Colo38 and NMB7 cells (5 x 10⁵ in 100 μl of PBS supplemented with 0.2% BSA and 0.02% NaN3) with dilutions of antisera for 1 h at 4°C. Then cells were washed six times and incubated with 100 μl of 1:200 dilution of fluorescein isothiocyanate–labeled goat anti-rabbit IgG. Thereafter, cells were washed three times with PBS-BSA-Na₂CO₃, and the autoradiographs were analyzed on a FACScan (Becton Dickinson). To determine the specificity of immune sera that bind to MPG⁺ cells over MPG⁻ cells, an absorption experiment was carried out as described (39). For this, MPG⁺ Meljur and MPG⁻ NMB7 cells were fixed with 1% paraformaldehyde for 30 min at room temperature and washed. The IM32 immune rabbit serum (100 μl of 1:50 dilution in PBS-BSA-Na₂CO₃ buffer) was incubated with these fixed Meljur and NMB7 cells for 2 h and washed with the same buffer.

The degree of absorption was determined by IIF analysis on Colo38 cells using serum before and after absorption on Meljur and NMB7 cells. The inhibition assay to map determinants recognized by different antibodies was performed by adding 125I-labeled MEM136 mixed with 25 μl of different dilutions of cold antibodies to Colo38 melanoma cells fixed on microtitre plates. At the end of a 16-h incubation at 4°C, plates were washed ten times with PBS, pH 7.4, and bound radioactivity was counted in a gamma counter. The results are expressed as a percentage of inhibition of binding of mAb MEM136 to Colo38 cells in the presence of control or immune sera, as compared to binding in the presence of preimmune sera.

Antibody Binding Assay with Extracellular Matrix or Components. Binding capability of antibodies to ECM components was determined in a direct binding RIA. Matrigel (Collaborative Research Inc., Bedford, MA), which is composed of collagen type IV, laminin, heparan sulfate, and a trace amount of chondroitin sulfate proteoglycan, was diluted 1:20 in serum-free Dulbecco's minimal essential medium, added (100 μl/well) to polystyrene plate (Dynatech Laboratories Inc., Chantilly, VA), and left at room temperature until the contents dried. Plates were washed with PBS to remove any unbound materials and blocked with 1% BSA in PBS for 1 h. Thereafter various dilutions of Ab2s (0.5% BSA) were added in different concentrations to the ECM-coated plates and incubated overnight at 4°C. After incubation, the plates were washed, and bound radioactivity was measured in a gamma counter.

Matrix Invasion Assays. The capacities of antibodies to modulate tumor cell invasion were evaluated using two types of in vitro assays. Tumor cell invasion across a basement membrane matrix barrier was evaluated using a minor modification of the assay described earlier (40). The 8.0-μm pore barrier membrane of each 6.5-mm Transwell insert (Costar, Cambridge, MA) was impregnated with 100 μl of 1:20 dilution of Matrigel basement membrane matrix extract (Collaborative Research, Inc.). Fifty thousand monodispersed tumor cells were added to the upper chambers of the two chamber culture units in 0.2 ml of complete medium. Colonies which developed in the lower well after 3 days of culture were washed, fixed in methanol, and stained with hematoxylin for counting.

The ability of the anti-idiotype antibodies to inhibit tumor cell invasion across the basement membrane matrix barrier was determined using an assay similar to that described above (39). The 8.0-μm pore barrier membrane of each 6.5-mm Transwell insert (Costar, Cambridge, MA) was impregnated with 100 μl of a 1:20 dilution of Matrigel basement membrane matrix extract (Collaborative Research, Inc.). Fifty thousand monodispersed tumor cells were added to the upper chambers of the two chamber culture units in 0.2 ml of complete medium. Colonies which developed in the lower well after 3 days of culture were washed, fixed in methanol, and stained with hematoxylin for counting.

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invasion was assessed by measuring the ability of Colo38 and NMB7 cells to invade collagen I gels complexed with chondroitin sulfate or heparan sulfate as described previously (41, 42). Briefly, 2-ml aliquots of rat type I collagen were mixed with 300 μl setting/neutralizing solution, and the mixture was added to 10 × 35-mm tissue culture dishes. Gels were allowed to set for 2 h at room temperature. The gels were further preincubated at 37°C in a CO2 incubator with 2 ml complete medium before cells were added. Gel cultures were seeded with 1.5 × 10⁵ tumor cells and fed every other day. Test antibodies were added to the culture media at 30 μg/ml. The number of cells invading the gel was counted on day 4 using phase-contrast microscopy with the dish placed on top of a standard grid. After the single cells within the gel had been counted, nuclear counts of all the cells in the culture were obtained. The ratio of single invading cells to total cells within the culture was derived and normalized to the control culture value for that experiment. This ratio was designated the “invasion index.”

RESULTS

Immunohistological Studies with MEM136. Immunohistological studies were performed to determine the tumor specificity and normal tissue cross-reactivity with MEM136. The data demonstrate that MEM136 detects an antigen associated with melanoma; virtually no cross-reactivity was found with the normal tissues tested except prostate and myometrium of the uterus. MEM136 also reacted with smooth muscles but did not bind to skeletal or cardiac muscles. In general mAb MEM136 reacted with normal skin tissues and nevi in a manner similar to that of other anti-MPG mAbs (7). In normal skin mAb MEM136 stained the basal squamous islands. The mAb MEM136 consistently exhibited linear staining of the plasma membrane. Nevi differed in their staining intensity depending on the location in the skin, with dermal nevi exhibiting a diffuse weak cytoplasmic staining, while nevi with junctional activity showed the intense granular stain characteristic of malignant melanoma cells.

MEM136 Recognizes MPG Antigen. Indirect immunoprecipitates obtained by reacting MEM136 with detergent lysates of Colo38 cells intrinsically labeled with [35S]methionine were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. A typical pattern is shown in Fig. 1. As seen in Fig. 1, Lane 2, mAb MEM136 immunoprecipitated the [35S]methionine-labeled components migrating at Ms 240,000, 250,000, and >400,000, which were characteristic of proteoglycan patterns obtained with 225.28S (Fig. 1, Lane 1); similar results have been obtained with other anti-MPG antibodies (7, 33). Fig. 1, Lane 3 demonstrates immunoprecipitation with MOPC21.

Fine Specificity of Monoclonal Anti-MPG Antibodies. The binding of 125I-MEM136 to the melanoma cell line Colo38 was examined in the presence of the following unlabeled anti-MPG or control antibodies: 225.28S; MEM136; 155.8; 149.53; 763.74T; TP41.2; and MOPC21. As demonstrated in Fig. 2A, only unlabeled MEM136 efficiently inhibited the binding of 125I-MEM136 to Colo38. The lack of inhibition of 125I-MEM136 to Colo38 cells with antibodies 225.28S, 155.8, 149.53, 763.74T, TP41.2, or MOPC21 demonstrated that MEM136 binds to an antigen associated with melanoma; virtually no cross-reactivity was found with the normal tissues tested except prostate and myometrium of the uterus. MEM136 also reacted with smooth muscles but did not bind to skeletal or cardiac muscles. In general mAb MEM136 reacted with normal skin tissues and nevi in a manner similar to that of other anti-MPG mAbs (7). In normal skin mAb MEM136 stained the basal squamous islands. The mAb MEM136 consistently exhibited linear staining of the plasma membrane. Nevi differed in their staining intensity depending on the location in the skin, with dermal nevi exhibiting a diffuse weak cytoplasmic staining, while nevi with junctional activity showed the intense granular stain characteristic of malignant melanoma cells.

Monoclonal Ab2s Recognize an Idiotope on MEM136. Splenocytes from A/J mice immunized with MEM136 (γ1,3) were hybridized with SP2/0 cells, and the resulting hybridomas were tested for their specificity for MEM136. The binding of different concentrations of these purified anti-id mAb IM04, IM06, IM08, IM09, IM13, IM19, IM20, and IM32 to MEM136-
Ab2s are binding site idiope specific. A. inhibition of binding of 125I-MEM136 to melanoma cells (Colo38) in the presence of different concentrations of IM04, IM08, and IM32. B. same study in the presence of different concentrations of IM06, IM09, IM13, IM19, and IM20. A known concentration of IC5 was used as a control in both studies. Points, one of three experiments.

F(ab')2 fragments was determined by ELISA. As a control, an unrelated anti-id mAb IC5 was included. All the anti-id mAbs, except IC5, only bound to mAb MEM136-F(ab')2 fragments and not to the isotype-matched control MOPC21-F(ab')2 (data not shown).

To determine the site specificity of these anti-idiotypic antibodies, the binding of 125I-MEM136 to Colo38 cells was measured in the presence of different concentrations of various Ab2s (Fig. 3). Based on their ability to inhibit this interaction, these Ab2 antibodies were divided into two distinct groups. Group 1, consisting of mAbs IM04, IM08, and IM32, required approximately 1 μg/ml to produce a 50% inhibition in the binding of 125I-MEM136 to Colo38, and all produced 80% inhibition at an antibody concentration of 3 μg/ml (Fig. 3A). Group 2, consisting of mAbs IM06, IM09, IM13, IM19, and IM20, required an antibody concentration of at least 2 μg/ml to achieve 50% inhibition of binding, and none of these achieved 80% inhibition at any antibody concentration tested (Fig. 3B). Binding inhibition by both groups appeared to be specific since an unrelated mAb2 (IC5) did not affect the binding of MEM136 to Colo38. Furthermore, mAb2s against mAb MEM136 did not inhibit binding of mAb C281 (anti-disialoganglioside mAb) to Colo38 cells (data not shown). Antibodies IM32 (group 1) and IM06 (group 2) were chosen for further characterization.

These results suggest that the idiotopes recognized by some of the Ab2s in group 1 are more closely related to the antigen combining site of mAb MEM136 than the Ab2s in group 2.

Ab2 Recognizes a Private Idiotypic Determinant on MEM136. Idiotypic relationships among anti-MPG mAb1s 225.28S, MEM136, 155.8, 149.53, 763.74T, and TP41.2 were analyzed in a competition assay (data not shown), where the binding of 125I-MEM32 or 125I-MIM6 to bound MEM136 was determined in the presence of various concentrations of different Ab1s. MEM136 effectively inhibited the binding of either labeled Ab2 to MEM136, producing 80% inhibition of 125I-MIM32 binding to MEM136 with 0.5 μg/ml of MEM136 (data not shown). Antibodies 155.8 or 225.28S were ineffective even at concentrations of 10 μg/ml. None of the other Ab1 antibodies was effective. Approximately 80% inhibition of binding of 125I-MIM6 to MEM136 (data not shown) was attained with MEM136 at a concentration of 2.5 μg/ml. A similar concentration of 225.28S produced less than 10% inhibition. No significant inhibition was obtained with 155.8, and none of the other Ab1 antibodies was effective. These data demonstrate that IM06 and IM32 recognize private idiotopes only on the immunizing mAb MEM136 molecule.

Ab2s May Share a Determinant but Differ in Their Fine Specificity. The spatial relationship of idiotopes recognized by various Ab2s was analyzed in a competition assay, where the binding of 125I-Mim32 (group 1) or 125I-MIM06 (group 2) to MEM136 was studied in the presence of different concentrations of various Ab2s. A known concentration of IC5 was used as a control in both studies. Points, one of three experiments.

Antibody Concentration (μg/ml)

Fig. 4. Ab2s differ in fine specificity. Each well of polyvinyl plates was coated with 50 ng of MEM136 in PBS and incubated overnight at 4°C. Plates were blocked with 1% BSA in PBS for 1 h, and the inhibition of 125I-MM32 binding to MEM136 was studied in the presence of different concentrations of various Ab2s. At 12 ng of different Ab2s, a range of inhibition of 125I-MIM32 binding to MEM136 of 0–50% was observed. Similar results were observed when 125I-MIM06 was used as a radioligand. Points, one of three experiments.
MEM136 was determined in the presence of different concentrations of unlabeled Ab2s. As shown in Fig. 4, the Ab2s displayed a broad range of inhibitory activity. When iodinated IM32 was used as a ligand, 50% inhibition of binding of $^{125}\text{I-IM32}$ to MEM136 was achieved with $<2\,\mu g/ml$ of IM04, IM06, IM08, and IM32. Other Ab2s required higher concentrations to produce 50% inhibition of binding of $^{125}\text{I-IM32}$ to MEM136. Similar results were obtained when IM06 was used as a radioligand (data not shown). These results suggest that all Ab2s inhibited the binding of $^{125}\text{I-IM06}$ or $^{125}\text{I-IM32}$ to MEM136 in a dose-dependent fashion and that the idiotope(s) recognized by these Ab2s are spatially linked or overlap with each other only slightly.

Analysis of the Variable Region of the Anti-Idiotypic mAbs. Immunochemical analysis was performed to determine the variability of the V-gene usage of the Ab2s. All eight Ab2s had a pH between 6.5 and 7.6 (Fig. 5) and comprised between two and four components. The spectrotype of each Ab2 was different, except that IM06 and IM09 were similar to each other, as were IM13 and IM32. This suggests a difference in the variable region polypeptide sequence and/or carbohydrate moiety.

Serological analysis was performed to corroborate the spectrotype analysis data. Xenogeneic antisera, obtained following Ab2 immunization of rabbits, were analyzed, thus allowing detection of contributions by both framework and complementary determining region determinants. As shown in Fig. 6, each antisera, containing Ab3 antibodies, inhibited the binding of the various anti-idiotype mAb2s to a different extent. No distinct association between binding site specificity (group 1 or 2) of the various Ab2s and the ability of the induced Ab3s to inhibit the binding of $^{125}\text{I-Ab2s}$ to MEM136 was observed. For example, the binding of $^{125}\text{I-IM06}$, $^{125}\text{I-IM13}$, $^{125}\text{I-IM20}$, and $^{125}\text{I-IM32}$ to MEM136 was variously inhibited by the different Ab2 immune sera. Similar results were obtained when the binding of $^{125}\text{I-IM04}$, $^{125}\text{I-IM09}$, $^{125}\text{I-IM13}$, and $^{125}\text{I-IM20}$ to MEM136 were studied in the presence of different Ab2 immune sera.

Immunoblotting of MEM136 Using Monoclonal Anti-Idiotypes. Idiotypes may be present on the isolated heavy or light chains of immunoglobulins or result from the association of both chains. MEM136 was reduced, and its heavy and light chains were electrophoretically resolved and blotted with various anti-idiotype antibodies. Of the nine anti-idiotype antibodies tested, none reacted with isolated heavy or light chains. When MEM136 was electrophoretically resolved and blotted under nonreducing conditions, all nine anti-idiotypic antibodies showed strong reactivity. A representative immunoblotting experiment is shown using IM32 and IM06 mAb2 (Fig. 7).

Induction of Anti-MPG Humoral Responses. Sera from rabbits immunized with eight mAb2s were tested for the presence of anti-MPG antibodies after two immunizations. Our goal is to identify anti-idiotype antibodies which can induce strong antitumor immune response (Ab1') after minimal immunization. Only IM06 and IM32 induced antitumor antibodies (Ab1') detected in both ELISA and IIF analysis. We have shown our IIF data demonstrating the binding of IM32 and IM06 immune sera preferentially to MPG~ Colo38 cells over MPG~ NMB7 cells (Fig. 8, A-D). As a control, we have included IIF binding with MOPC21 immune rabbit sera (Fig. 8, E and F).

Modulation of Tumor Cell Invasion. At a concentration of 30 $\mu g/ml$ MEM136 inhibited invasion of the Matrigel basement membrane matrix by Colo38 cells to about half of that shown by control cultures (Table 1). Ab2s raised to this antibody showed varying effects. IM06 was the most inhibitory, followed by IM32, while IM08, IM09, and IM20 facilitated invasion. When tested against the invasion of a simplified interstitium-like matrix, a complex gel of collagen type I and chondroitin sulfate, all the Ab2s were either clearly inhibitory toward invasion by Colo38 cells (IM04, IM32, IM20) or showed marginal inhibitory effects (IM08, IM06, IM09). When the matrix was modified to substitute the basement membrane glycosaminoglycan, heparan sulfate, for chondroitin sulfate, IM32 and IM06 still exhibited inhibitory properties while IM20 had no effect. IM06 and IM32 had no effect on invasion of the complex collagen I gels by the MPG~ NMB7 cell line (Table 1). Furthermore, pretreatment of melanoma cells with MEM136 was shown to block the migration and invasion of melanoma cells. Inhibition of invasion by mAb MEM136 and mAb2s is specific, since anti-class I mAb TP25.99 did not have any effect on the melanoma cell behavior in the invasion assay. These data demonstrate that MEM136 detects an MPG epitope which is critical for the melanoma cell invasion of ECM.

Reactivity of Anti-Ids to the Matrix. To determine a possible mechanism by which IM32 and IM06 inhibit tumor cell invasion, we performed direct binding RIA of $^{125}\text{I-anti-ids}$ to the matrix and to the MPG~ Colo38 cells and MPG~ NMB7. The results shown in Fig. 11 demonstrate the binding of various

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![Fig. 5. Isoelectric focusing of Ab2s. The pls of the Ab2s were determined using a pH 3–10 isoelectric focusing gel as described in “Materials and Methods.” Lane 1, IM04 (γ1,λ); Lane 2, IM06 (γ1,λ); Lane 3, IM08 (γ1,λ); Lane 4, IM09 (γ1,λ); Lane 5, IM13 (γ1,λ); Lane 6, IM20 (γ1,λ); Lane 7, IM30 (γ20,λ); Lane 8, IM32 (γ1,λ).](source)
Fig. 6. Inhibition by rabbit anti-anti-idiotype antisera of the binding of $^{125}$I-Ab2s to MEM 136. Ab3 antisera elicited by IM04, IM06, IM08, IM09, IM13, IM20, and IM32 were incubated with $^{125}$I-IM06 (A), $^{125}$I-IM13 (B), $^{125}$I-IM20 (C), and $^{125}$I-IM32 (D) on MEM136-coated microtiter plates. After overnight incubation, the plates were washed, and bound radioactivity was measured in a gamma counter. Similar results were obtained in two independent experiments.

Fig. 7. Immunoblotting of mAb MEM136 using anti-id mAb. The mAb2s IM32 and IM06 react only with whole MEM136 (A, Lanes 1 and 3) and not with isolated H or L chain (A, Lanes 2 and 4). B, representative control blotting stained with Ponceau S demonstrating that whole IgG (Lane 1) and separated H and L chain (Lane 2) are present under the conditions used in our experiment.
to an isotype-matched mouse myeloma protein MOPC21- F(ab')2. All monoclonal anti-id recognize private idiotopes, since they only react with immunizing mAb MEM136 and not against a panel of several monoclonal anti-MPG antibodies. One might argue that since the mAb MEM136 recognizes an epitope different from that recognized by other anti-MPG mAbs, it is likely that anti-id mAb generated against MEM136 will recognize only the immunizing mAb MEM136. However, there are reports in the literature that mAb having different specificities cross-react idiotypically (56). These idiotypes are termed cross-reactive or regulatory idiotypes. Our results indicate that none of our mAb2s is reactive against the cross-reactive or regulatory idiotopes. The idiotopes defined by eight mAb2s require the association of the heavy and light chains of mAb MEM136 for their expression, since they do not react with isolated heavy and light chains in Western blotting. Idiotype mapping experiments indicate that idiotopes recognized by mAb2s on mAb MEM136 are overlapping since all eight mAb2s inhibited the binding of 125I-MEM136 to Colo38 cells. However, mAb2s differ in their ability to inhibit 125I-MEM136 binding to Colo38 cells. Ab3s elicited by the eight mAb2s contain antibodies with cross-reacting and private idiotypic specificities as determined by cross-inhibition studies where binding of 125I-IM04, 125I-IM06, 125I-IM08, 125I-IM13, 125I-IM19, 125I-IM20, and 125I-IM32 to mAb MEM136 was studied.
in the presence of Ab3 containing sera from various mAb2 immunized rabbits. The differential inhibition of $^{125}$I-MEM136 and mAb2 interaction by different anti-anti-idiotypic sera is not due to the presence of anti-mouse immunoglobulin antibodies, because rabbits immunized with different mAb2s produced an equivalent amount of anti-immunoglobulin antibodies (data not shown). Our results also indicate that with certain mAb2s, Ab3s against heterologous Ab2 are better inhibitors of the Ab2-Ab1 reaction than the Ab3 against the homologous Ab2. For example, the binding of $^{125}$I-IM06 and $^{125}$I-IM20 to mAb MEM136 inhibited better with heterologous mAb2 immune sera (Fig. 6). This heteroclitic response could be due to the fact that some cross-reactive idiotopes which exist on Ab2s can induce stronger Ab3 response in certain conformation. For example, Ab3s induced against a cross-reactive epitope can be elicited with IM13 than by IM20. Therefore, $^{125}$I-IM20 binding to MEM136 could be better inhibited by IM13 immune sera than by the Ab3 sera induced by IM20. Alternatively, the heteroclitic reactivity patterns of the antisera may reflect variability in immune responses of immunized rabbits. With further regard to inhibition, studies using anti-anti-idiotypic anti-sera could be made of the affinity of the Ab2, which is unlikely since all Ab2s possess similar relative affinities for the mAb

MEM136.$^4$ Melanoma cell binding studies indicate that only IM06 and IM32 immune rabbit sera contain anti-MPG reactivity as determined by the ability of the antisera to bind preferentially to MPG$^+$ Colo38 cells over MPG$^-$ NMB7 cells. Furthermore, IM32 and IM06 immune sera inhibited the binding of $^{125}$I-MEM136 to Colo38 cells implying Ab1 and Ab1$'$ are recognizing similar epitopes. It should be emphasized that our goal was to identify the best internal image candidate that would induce relevant antitumor antibodies with minimal immunization. Therefore, it is logical to study the secondary immune responses rather than the tertiary and quaternary im-

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**Table 1** Effect of mAb MEM136 and mAb2s raised against it on matrix invasion by MPG$^+$ Colo38 melanoma cells and MPG$^-$ NMB7 neuroblastoma cells$^a$

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Cell line</th>
<th>Matrix tested</th>
<th>Collagen 1 + chondroitin sulfate (II)</th>
<th>Collagen 1 + heparan sulfate (II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM136</td>
<td>Colo38</td>
<td>47 ($P = 0.01$)$^a$</td>
<td>ND$^a$</td>
<td>ND</td>
</tr>
<tr>
<td>TP25.99</td>
<td>Colo38</td>
<td>1.1 ($P = 0.45$)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IM04</td>
<td>Colo38</td>
<td>0.53 ($P = 0.14$)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IM08</td>
<td>Colo38</td>
<td>163 ($P = 0.15$)</td>
<td>0.86 ($P = 0.20$)</td>
<td>ND</td>
</tr>
<tr>
<td>IM32</td>
<td>Colo38</td>
<td>71 ($P = 0.06$)</td>
<td>0.49 ($P = 0.07$)</td>
<td>0.58 ($P = 0.03$)</td>
</tr>
<tr>
<td>IM06</td>
<td>Colo38</td>
<td>25 ($P = 0.008$)</td>
<td>0.81 ($P = 0.09$)</td>
<td>0.50 ($P = 0.019$)</td>
</tr>
<tr>
<td>IM09</td>
<td>Colo38</td>
<td>181 ($P = 0.15$)</td>
<td>0.85 ($P = 0.43$)</td>
<td>ND</td>
</tr>
<tr>
<td>IM20</td>
<td>Colo38</td>
<td>179 ($P = 0.11$)</td>
<td>0.46 ($P = 0.10$)</td>
<td>0.92 ($P = 0.37$)</td>
</tr>
<tr>
<td>IM06</td>
<td>NMB7</td>
<td>1.14 ($P = 0.45$)</td>
<td>1.06 ($P = 0.42$)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Data are averages of between 2 and 4 runs containing 4 (Matrigel) or 2 (collagen I gels) replicates for each data point. All affinity purified antibodies were present at 30 gg/ml.  
$^b$ BM, basement membrane.  
$^c$ II, invasion index calculated as described in the text.  
$^d$ Statistical analysis was carried out using Student's $t$ test applied to the log 10 transferred data values; pooled weighted variance of the data shown (log 10 scale) = 0.0927.  
$^e$ ND, not done.
mune responses, although all rabbits were boosted three times for different purposes.

MPG molecules are believed to be pivotal in potentiating the metastatic properties of melanoma (4, 12, 14, 15, 25). During the process of invasion and metastasis, it is necessary for tumor cells to traverse the ECM. The basement membranes within the ECM are composed mainly of collagen type IV, meshed laminin, entactin, fibronectin, and proteoglycans. A substantial body of evidence suggests a close correlation between extracellular matrix degradation and tumor cell invasion and metastasis (42). Therefore, if tumor cells are prevented from binding to the ECM, metastasis may be inhibited. To this end, a humoral response can be induced against MPG to develop antibodies which will act as antagonists and interfere with the binding of melanoma cells to the basal lamina or the basement membrane. In the Matrigel assay, MEM136 inhibited melanoma cell invasion. Since this matrix contains only a trace of chondroitin sulfate proteoglycan, it suggests that the antibody is interacting directly with the tumor cell membrane to alter its invasive characteristics. The mAb MEM136-mediated inhibition of melanoma cell invasion is specific since mAb TP25.99, directed against the class I molecule, did not demonstrate any significant inhibition of melanoma cell invasion in our assay. Furthermore, IM32 and IM06 did not have any effect on the behavior of MPG-NMB7 cells in the same invasion assay. Thus, the MPG epitope being studied may be biologically important in tumor cell invasion.

We also investigated the interaction between the melanoma cell and the basement membrane in the presence of various mAb2s. We reasoned that some mAb2s may contain an image of the MPG epitope which is important for the tumor cell-ECM interaction required for melanoma cell invasion. In both cell migration and invasion assays, we observed an inhibitory effect of mAb IM32 and IM06. The mAb2s, if they represent molecular images of the membrane proteoglycan epitopes, should be potentially active in modulating tumor cell invasion in vitro. When tested against the invasion of a basement membrane matrix, mAb IM32 and IM06 were inhibitory while mAb IM20, IM09, and IM08 clearly facilitated invasion. The Ab2 IM20 is mechanistically interesting since it facilitates melanoma invasion of basement membrane and inhibits invasion of the simplified interstitium-like matrix of collagen type I containing chondroitin sulfate. However, it has no effect on a matrix containing the interstitial component, collagen type I, and the basement membrane glycosaminoglycan, heparan sulfate. These findings suggest that basement membrane components other than the glycosaminoglycans are involved in modulation of invasion of this matrix. Likely candidates are laminin and collagen type IV. Similar results have been reported earlier (41). To define a mechanism by which mAb2s modulate melanoma cell invasion, direct binding of $^{125}$I-Ab2 to Matrigel or to Colo38 cells was assessed. Our data suggest that only IM06 and IM32 can bind to Matrigel. None of the Ab2s bound to Colo38 cells (data not shown). The binding of $^{125}$I-IM06 and $^{125}$I-IM32 to Matrigel is weak because only <1% of the counts added bound to the gel. This weak binding could be due to the fact that only a minor component of the ECM acts as a receptor for the MPG antigen surrogate, IM32 and IM06. The binding of $^{125}$I-IM32 and $^{125}$I-IM06 to Matrigel is specific, since various other mAb2s generated against the same mAb MEM136 did not demonstrate binding to the same gel, including the mAb2s which shared the enhancing effect of Colo38 cell invasion. Our data suggest that anti-id mAb IM32 and IM06 may bind to a receptor of MPG antigen expressed on melanoma cells which is partly involved in the melanoma cell-ECM interaction required for tumor cell invasion. However, we could not demonstrate any inhibition of binding of $^{125}$I-IM32 or $^{125}$I-IM06 to Matrigel by cold mAb MEM136 (data not shown). Our finding does not prove that the binding of two mAb2s is not occurring via the internal image of the antigen present on anti-idiotypic antibody. The lack of inhibition of binding of mAb2 to Matrigel by mAb1 could be due to the difference in affinity where mAb2 has much higher affinity for Matrigel than the Ab1 has for the Ab2 idiotope. Therefore, a reciprocal inhibition experiment is needed. Studies are being performed to determine which component of the Matrigel is involved in such binding.

Another intriguing point for discussion is why some of the Ab2s generated against MEM136 enhanced melanoma cell invasion in absence of binding to Matrigel. It is possible that the enhancing antibodies are binding to a component which is present in very small amounts in the Matrigel and that our binding assay could not detect such a low level of binding. Another possibility is that enhancing antibody could be sticky antibody, which is unlikely as all the antibodies are $\gamma_1, \kappa$ and do not bind to plain plastic or plastic coated with 1% BSA or FBS (data not shown). However, we are repeating our binding assay using $^{125}$I-mAb2 to the Matrigel composed of individual ECM components to define precisely the molecule responsible for binding.

Several possible mechanisms can be offered to explain the observed effects of the melanoma cell invasion by IM32 and IM06 anti-id mAb. Experimental evidence suggests a close relationship between extracellular matrix degradation and tumor cell invasion and metastasis (22–24). Tumor cells may not bind to the substrate in the presence of the MPG image antibody and therefore will no longer degrade the matrix, thus inhibiting invasion. Furthermore, the presence of the MPG image antibody may prevent cell-to-cell interactions and thereby inhibit colony formation. Finally, the MPG image antibody might directly inhibit cellular mobility by binding to cell receptors, modifying the avidity of receptor:ligand binding, or by modifying receptor release mechanisms required during cellular movement.

To summarize, we have generated a library of both binding site-related and -unrelated mAb2s which are immunologically distinct. Our results suggest that internal image anti-idiotypic antibodies such as IM06 and IM32, which are universally inhibitory toward melanoma invasion of matrices and at the same time induce anti-MPG humoral responses in rabbits, represent potential vaccine candidates.

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Monoclonal Anti-Idiotypic Antibodies to Human Melanoma-associated Proteoglycan Antigen: Generation and Characterization of Anti-Idiotype Antibodies

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