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Murine monoclonal anti-idiotope antibody breaks unresponsiveness and induces a specific antibody response to human melanoma-associated proteoglycan antigen in cynomolgus monkeys

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ABSTRACT **The mouse monoclonal antibody MEM136 (mAb1) is directed against an epitope on human melanoma-associated proteoglycan antigen (MPG). This epitope is also present on various normal human and subhuman tissues. A monoclonal murine anti-idiotope (anti-Id) antibody (mAb2), designated I-Mel-2, was generated against MEM136 and used as a surrogate antigen for the MPG molecule. I-Mel-2 was tested in cynomolgus monkeys (*Macaca fascicularis*) for its ability to induce anti-MPG humoral responses. All monkeys immunized with Ab2 developed specific anti-anti-idiotype (Ab3) responses that were capable of inhibiting binding of Ab2 to Ab1. Furthermore, I-Mel-2 immune monkey serum contained anti-MPG antibodies (Ab1′) that bound to MPG-positive but not to MPG-negative melanoma cell lines. Monkeys immunized with Colo38 melanoma cells (membrane-bound MPG antigen) did not contain anti-MPG antibodies that inhibited the binding of two distinct anti-MPG mAb ^{125}I-labeled MEM136 or ^{125}I-labeled 225.28 to Colo38 cells. The induction of anti-MPG responses in monkeys did not cause any apparent side effects in animals, despite the fact that the MPG antigen is expressed by many normal tissues. The affinity-purified, I-Mel-2 idiotype-specific, Ab3 immunoprecipitated MPG antigen from melanoma cells. Furthermore, the I-Mel-2-induced Ab3 inhibited melanoma cell invasion in an *in vitro* assay, implying that these antibodies have biological significance.**

Melanoma-associated proteoglycan (MPG) antigen is expressed on the cell surface of the majority of melanomas and in lesser amounts on various normal tissues, including keratinocytes and endothelial cells (1–3). The MPG molecule is thought to play an important role in determining the invasive and metastatic potential of melanoma cells, and monoclonal antibodies (mAb) to the MPG molecule have been shown to block invasion, tumor growth inhibition *in vivo*, and tumor colony formation *in vitro* (1, 4–6). These studies imply that an active induction of an anti-MPG humoral response in patients could be beneficial for controlling the spread and growth of melanoma cells.

Anti-idiotope (anti-Id) mAb (mAb2) have been used to define idiotopes on immunoglobulin molecules. Some Ab2 represent the internal image of the antigen and induce active immune responses against tumor-associated antigens in animal models (7–13). Moreover, animal studies have demonstrated that anti-Id antibodies can be used to break the state of unresponsiveness or tolerance that appears to exist with nominal antigens (14).

We have described earlier (15) the generation and characterization of an anti-Id mAb2, termed "I-Mel-2" (designated earlier as IM32), generated against MEM136. MEM136 is an anti-MPG mAb, directed against an epitope present on chondroitin sulfate proteoglycan (15), and because it demonstrated a significant antiinvasive property in an *in vitro* assay against melanoma cells, we used this antibody to generate anti-Id mAb as candidate immunotherapeutic agents. Earlier (16), we demonstrated that of eight anti-Id Ab2, only I-Mel-2 induced anti-MPG responses in mice and rabbits. In this study we have investigated the effect of I-Mel-2 on the induction of anti-MPG humoral responses in cynomolgus monkeys (*Macaca fascicularis*). These animals express human MPG crossreacting antigen in normal tissues defined by MEM136. Therefore, the immune response of subhuman primates is likely to be predictive of the ability of I-Mel-2 to induce an immune response in patients with melanoma.

MATERIALS AND METHODS

Cell Lines. The human melanoma cells Colo38, Meljur, and 397, which express the MPG antigen (MPG-positive), were used for the detection of anti-MPG responses. They were grown in RPMI 1640 medium supplemented with 10% calf serum, 1% glutamine, and 10 μg of gentamycin sulfate per ml. The NMB7 neuroblastoma cells (a kind gift from S. K. Liao, McMaster University, Hamilton, ON, Canada), human melanoma MeWo (A16) cells, and MCF7 mammary carcinoma cells, which do not express MPG (MPG-negative), were grown in the same medium and were used as negative controls. The MeWo (A16) cell line was obtained from J. Roder (Toronto University). The 397 cell line was obtained from M. Lotze (Pittsburgh Cancer Center).

Ab1, Ab2, and Conventional Antisera. mAb MEM136 (γ1, κ), I-Mel-2 (γ1, κ), and 5A8 (γ1, κ) were purified on a protein A-Sepharose 4B column. The isotype-matched mouse myeloma protein MOPC21 (γ1, κ) (Bionetics Research Institute) was used as control. MEM136 recognizes an epitope on the MPG molecule and I-Mel-2 is an anti-Id antibody specific for an MEM136 idiotope (15). mAb2 5A8, used as a control, is a mouse anti-Id antibody directed against human polyclonal anti-gp120 antibodies (C. Y. Kang, unpublished observation). The anti-HLA class II mAb 05/13 (γ2a, κ), used as a control, was provided by S. Ferrone (New York Medical College).

Immunization of Monkeys. Cynomolgus monkeys (three per group) received six intramuscular injections of I-Mel-2 mixed with Syntex adjuvant formulation SAFm (17). Control monkeys were immunized with anti-Id mAb 5A8 mixed with SAFm. Animals receiving antibody were given 2.5 mg per injection. Two monkeys (in each group) were immunized with Colo38 and MCF-7 cells (5×10^6 cells per monkey) following

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Abbreviations: MPG, melanoma-associated proteoglycan; I-Mel-2-Ab3, affinity-purified, I-Mel-2 idiotype-specific Ab3 purified from I-Mel-2-immunized monkey serum; 5A8-Ab3, affinity-purified, 5A8 idiotype-specific Ab3 purified from 5A8-immunized monkey serum; mAb, monoclonal antibody(ies); anti-Id, anti-idiotope.

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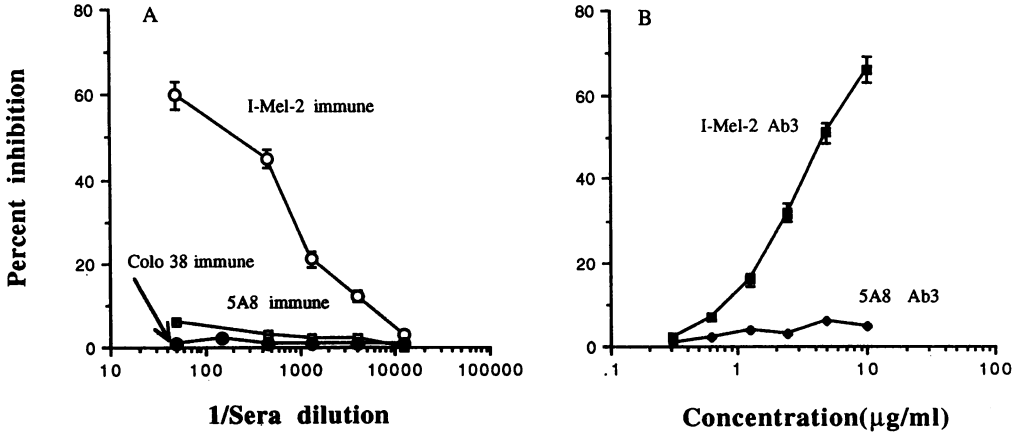

FIG. 1. Induction of anti-anti-idiotype (Ab3) response. (*A*) The total anti-anti-idiotype (Ab3) response in serum of monkeys immunized with I-Mel-2 (○) was analyzed by a homologous inhibition assay, in which binding of ^{125}I-labeled MEM136 to F(ab')$_2$ fragment of I-Mel-2 was studied at different dilutions of immune serum after absorption on normal mouse IgG. Serum from monkeys immunized with mAb 5A8 (□) was used as a control. No inhibition of ^{125}I-labeled MEM136 to I-Mel-2-F(ab')$_2$ by Colo38 immune serum (●) was observed. The results are expressed as percentages of inhibition that are arithmetic means of triplicate determinations from three animals in each group. (*B*) Inhibition of binding of ^{125}I-labeled MEM136 to I-Mel-2-F(ab')$_2$ by various concentrations of unlabeled I-Mel-2-Ab3 (■). As a control, 5A8-Ab3 (◆) was used. The experiment was performed by adding either different dilutions of serum (*A*) or different concentrations of purified Ab3 (*B*) and ^{125}I-labeled MEM136, without prior incubation, to the microtiter plates coated with I-Mel-2-F(ab')$_2$ (100 ng per well). Incubation was carried out for 18 hr at 4°C; this was followed by washing and assay for radioactivity in a γ counter.

the same schedule. All injections were given at 2-week intervals. Monkeys were bled 7 days after each immunization.

Serological Assays. Indirect immunofluorescence was performed by incubating target cells (5×10^5 in phosphate-buffered saline supplemented with 0.2% bovine serum albumin and 0.02% NaN_3) with dilutions of antiserum or purified antibodies for 1 hr at 4°C as described (16). To detect monkey IgG bound to human cells, the staining was done with fluorescein isothiocyanate-labeled goat anti-human IgG (Becton Dickinson) and analyzed on a FACScan (Becton Dickinson) flow cytometer. The inhibition assay to map determinants recognized by different antibodies was performed as described (16).

The idiotypic response was analyzed by a radioimmunoassay as described (18). The binding of ^{125}I-labeled anti-anti-Id (I-Mel-2-Ab3) to melanoma cells was performed as described (19). To determine nonspecific binding, ^{125}I-labeled 5A8-Ab3 was used.

Immunochemical Methods. Indirect immunoprecipitation was performed as described (16).

Matrix Invasion Assays. The capacities of antibodies to modulate tumor cell invasion were evaluated using a "Matrigel" assay, the MPG-positive cell lines Colo38 and Meljur, and the MPG-negative MeWo cell line. Tumor cell invasion across the basement membrane matrix was evaluated using the methods described earlier (16, 20, 21).

RESULTS

Induction of Anti-Anti-Idiotype (Ab3) Response in Monkeys. To determine the immunogenicity of 1-Mel-2 in monkeys, we analyzed the Ab3 response with normal mouse IgG absorbed serum from monkeys immunized with 1-Mel-2 or control 5A8 obtained 7 days after the fourth immunization (4d7 serum). Fig. 1*A* demonstrates that the serum sample contains an Ab3 population, as shown by their ability to inhibit the binding of ^{125}I-labeled MEM136 to the F(ab')$_2$ fragment of I-Mel-2. Monkey serum immunized with mAb2 5A8 did not show any inhibition of binding of ^{125}I-labeled MEM136 to I-Mel-2. As shown in Fig. 1*A*, the IC_{50} of inhibition of MEM136 binding to I-Mel-2-F(ab')$_2$ was achieved at a serum dilution of 1:200. By comparing these data with a standard curve (Fig. 1*B*) of unlabeled affinity-purified Ab3 (i.e., I-Mel-2-Ab3, the Ab3 purified from I-Mel-2 immune monkey serum), the serum Ab3 concentration was determined. This was ≈800 μg/ml of serum from monkeys immunized with I-Mel-2. Fig. 1*A* also shows that the serum from monkeys immunized with Colo38 cells could not inhibit the binding of ^{125}I-labeled MEM136 to I-Mel-2-F(ab')$_2$, indicating that monkey anti-Colo38 immune serum does not contain a detectable idiotype shared by MEM136 that interferes with the Ab1–Ab2 interaction.

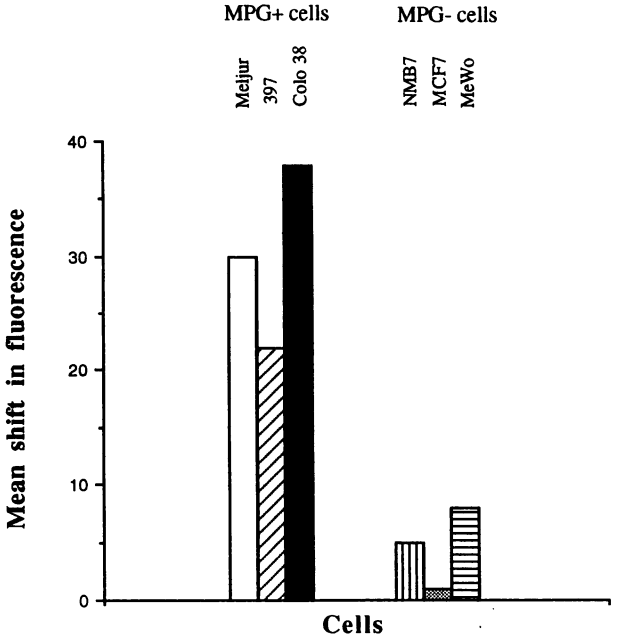

FIG. 2. Reactivity of immune serum to MPG-positive (MPG+) and MPG-negative (MPG−) cells: anti-MPG response in serum of monkeys immunized with mAb I-Mel-2 as determined by indirect immunofluorescence analysis. Colo38 (MPG+, ■), Meljur (MPG+, □), 397 (MPG+, ▨), MeWo (MPG−, ▤), NMB7 (MPG−, ▥), and MCF7 (MPG−, ▩) cells were incubated with different dilutions of serum, washed, and subsequently labeled with fluorescein-conjugated goat anti-human IgG. Data were obtained from a representative monkey with serum dilutions of 1:500. The mean shift in fluorescence was determined by subtracting the shift obtained with preimmune serum from the shift obtained with postimmune serum. No shift was observed with control mAb 5A8 immune serum.

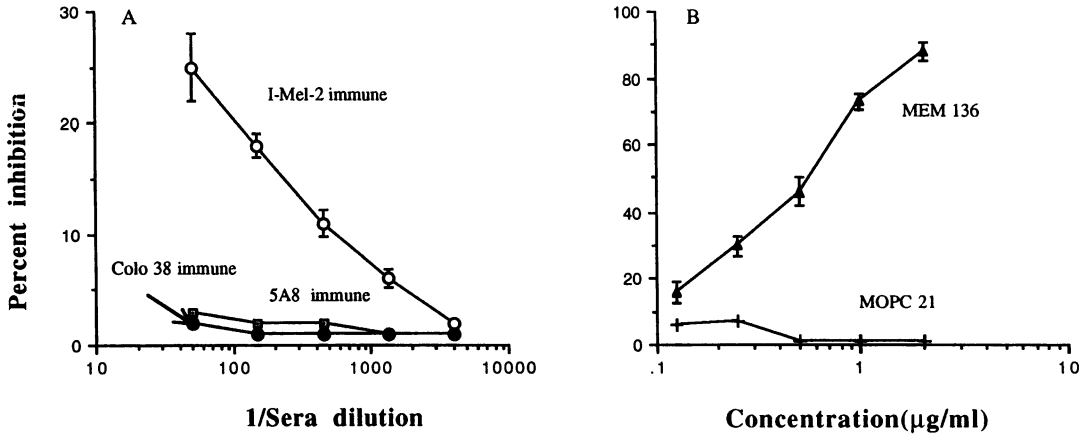

FIG. 3. Ab1′ response in immune serum. (*A*) The Ab1′ response in serum of monkeys immunized with mAb I-Mel-2 (○) was analyzed by inhibition of binding of ^{125}I-labeled MEM136 to Colo38 cells using different dilutions of serum. Results are expressed as percentages of inhibition, which are arithmetic means of triplicate determinations from three animals in each group. Serum from primates immunized with 5A8 (□) was used as a control. No inhibition of ^{125}I-labeled MEM136 binding to Colo38 by Colo38 immune serum (●) was observed. (*B*) A standard inhibition curve was derived from an inhibition assay, in which binding of ^{125}I-labeled MEM136 to Colo38 was determined in the presence of different concentrations of unlabeled mAb MEM136 (▲) or the isotype-matched control MOPC21 (+). The experiment was carried out by adding either different dilutions of serum (*A*) or different concentrations of mAb MEM136 (*B*) and ^{125}I-labeled MEM136, without prior incubation, to the glutaraldehyde-fixed Colo38 plates. Incubation was carried out for 18 hr at 4°C; this was followed by washing and assay for radioactivity in a γ-counter.

Induction of Anti-MPG Response. To assess whether I-Mel-2 immune monkey serum bound specifically to MPG-positive melanoma cells, the MPG binding assay was performed by indirect immunofluorescence analysis using several MPG-positive and MPG-negative cell lines. Fig. 2 shows representative data from one monkey immunized with I-Mel-2 at a dilution of 1:500. The data indicate that I-Mel-2 induced antibodies that bound specifically to MPG-positive cells, Meljur, 397, and Colo38, since substantially less binding was observed with MPG-negative cells, NMB7, MCF7, and MeWo. Moreover, the control (5A8 in SAFm) serum did not bind to either Colo38 or NMB7 cells (data not shown). To compare the epitope specificity of Ab1 and the induced Ab1′, we tested whether I-Mel-2 immune serum could inhibit the binding of ^{125}I-labeled MEM136 to Colo38 cells (Fig. 3*A*). Approximately 25% inhibition of binding of ^{125}I-labeled MEM136 to melanoma cells was achieved specifically at a dilution of 1:50, suggesting that Ab3 antibodies reacting with the determinant defined by the Ab1, MEM136, were present in the anti-I-Mel-2 serum of cynomolgus monkeys, although in low concentration. By comparison with an inhibition curve obtained with known concentrations of MEM136 (Fig. 3*B*), it was estimated that 9.5 μg of Ab3 antibodies (≈1%) per ml of serum from monkeys immunized with I-Mel-2 had specificity similar to that of the MEM136 (Ab1) antibody. Furthermore, the Colo38 immune serum could not inhibit the binding of ^{125}I-labeled MEM136 to Colo38 cells (Fig. 3*A*) or ^{125}I-labeled 225.28 (a different anti-MPG mAb than MEM136) (19) binding to Colo38 cells (data not shown), indicating that monkey anti-Colo38 serum does not contain anti-MPG antibodies directed against MEM136 or 225.28 epitopes.

To confirm that Ab3 was responsible for MPG binding and to determine biological activity, these antibodies were purified from I-Mel-2 immune serum. Due to a limited supply of serum, Ab3 was purified from a pool of immune serum taken from three monkeys 7 days after the fourth immunization. The reactivity of Ab3 with MPG was indicated by SDS/PAGE analysis of antigens immunoprecipitated from Colo38 cells. As demonstrated in Fig. 4*A*, monkey Ab3 induced by I-Mel-2 immunoprecipitated antigens (lane 4) from MPG-positive Colo38 cells with the same size as those immunoprecipitated by the Ab1, MEM136 (lane 2). The immunoprecipitation was specific since 5A8-Ab3 (lane 3) did not precipitate any component from the melanoma cell lysate. The I-Mel-2-Ab3 immunoprecipitated very poorly components from a Colo38 melanoma cell lysate that had been immunodepleted with the MEM136 antibody (Fig. 4*B*, lane 2). Furthermore, neither I-Mel-2-induced Ab3 nor MEM136 immunoprecipitated any component from MPG-negative NMB7 cells (Fig. 4*C*). The fact that Ab1 and I-Mel-2-Ab3 recognize 250-kDa and >400-kDa molecular species from a melanoma cell lysate is in agreement with a previous report (22).

Finally, Fig. 5 demonstrates that I-Mel-2-Ab3 competed with mAb1 MEM136 to bind to Colo38 cells, and the Ab1-like antibody fraction was estimated to be 1.4% of the total Ab3 population. This is similar to values obtained with unpurified serum. On the other hand, the percentage of antibody molecules reacting with Colo38 cells was ≈4–8% in I-Mel-2-Ab3 antibodies (Table 1), implying only a fraction of the total anti-MPG antibodies contains specificity similar to mAb1.

Effects of Ab3 Antibodies on the Interaction of Colo38, Meljur, and MeWo Cells with Matrigel. The ability of I-Mel-

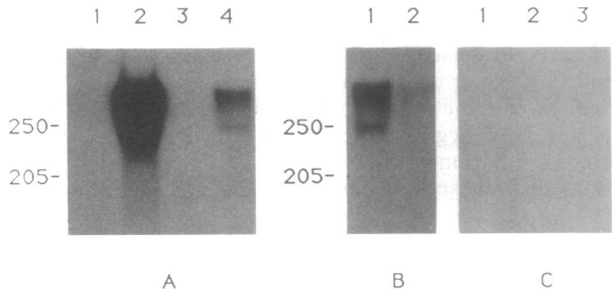

FIG. 4. Recognition of the same antigen by Ab1 and Ab3: SDS/PAGE analysis of antigens immunoprecipitated from Colo38 melanoma cells by anti-MPG mAb MEM136 and by 1-Mel-2-Ab3. Immunoprecipitations with anti-MPG mAb MEM136 (10 μg) (*A*, lane 2), the isotype-matched control MOPC21 (10 μg) (*A*, lane 1), 5A8-Ab3 (*A*, lane 3), and an extract immunodepleted with isotype-matched control MOPC21 (100 μg) (*B*, lane 1) were used as controls. An extract of ^{125}I-labeled Colo38 cells (*A*, lane 4) and an extract immunodepleted with anti-MPG mAb MEM136 (100 μg) (*B*, lane 2) were immunoprecipitated with I-Mel-2-Ab3 (100 μg). (*C*) NMB7 cell lysate corresponding to 5×10^7 cpm was allowed to react with MEM136 (lane 1), I-Mel-2-Ab3 (lane 2), and 5A8-Ab3 (lane 3). Autoradiograph film was exposed for 3 days before development. Sizes are indicated in kDa.

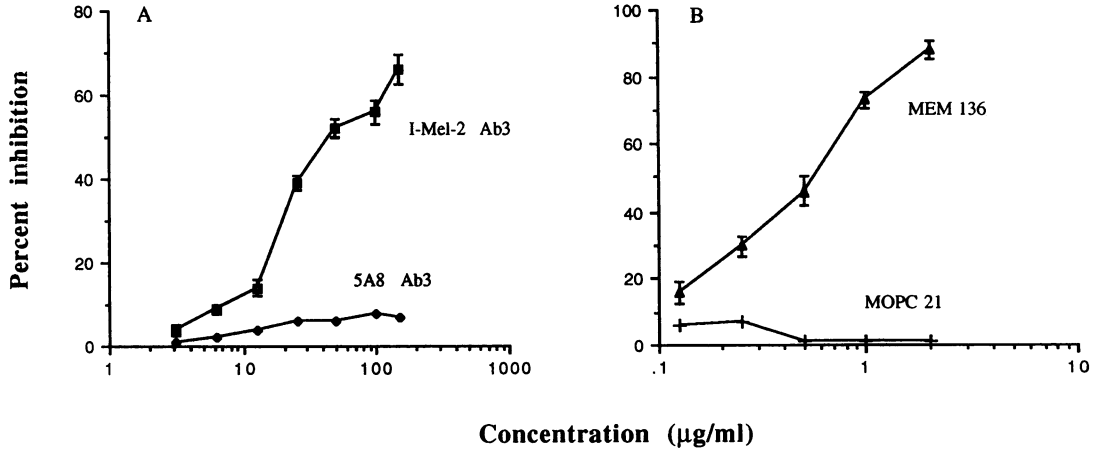

FIG. 5. Epitope specificity of Ab1 and Ab1′ in purified Ab3. (*A*) Percent inhibition of binding of ^{125}I-labeled MEM136 to Colo38 cells by I-Mel-2-Ab3 (■) is shown. The 5A8-Ab3 (◆) was used as a control. The experiment was done as described in the legend to Fig. 3. (*B*) Standard inhibition curve, in which the inhibition of ^{125}I-labeled MEM136 binding to Colo38 was determined in the presence of different concentrations of MEM136 (▲) and MOPC21 (+).

2-induced monkey Ab3 to inhibit Colo38 and Meljur cell invasion was tested in the Matrigel assay (Table 2). MEM136 was used as a positive control. MEM136 and I-Mel-2-Ab3 inhibited the invasion of Matrigel by MPG-positive Colo38 and Meljur cells at a final concentration of 30 μg/ml but had no effect on MPG-negative MeWo cells. Inhibition of invasion was specific since neither the MOPC21 myeloma protein nor the control 5A8-Ab3 had any detectable effect on Colo38 invasion. Furthermore, the mAb 05/13, specific for class II molecules present on Colo38, did not have any inhibitory effect on the invasive properties of these cells.

DISCUSSION

Considering the importance of the MPG molecules in determining its growth, invasiveness, and metastatic potential of melanoma cells, it is desirable to induce a humoral immune response against the MPG antigen for management of the disease (refs. 1 and 4 and discussed below). The data described here demonstrate that a mouse anti-Id mAb, I-Mel-2 made against a MPG-specific mAb, MEM136, can induce a specific anti-MPG humoral response in cynomolgus monkeys. An important feature of this work is that none of the monkeys suffered any apparent side effects associated with autoimmune syndromes. Our observation is significant especially in the field of tumor immunotherapy because an anti-tumor antibody response was induced in spite of the presence of MPG crossreacting antigen on normal monkey tissues. Due to a lack of direct evidence, it can only be speculated that I-Mel-2 can break unresponsiveness to the MPG antigen in non-human primates. Evidence to support this assumption comes from experiments in which monkeys immunized with cell-bound antigen—i.e., with melanoma cells (Colo38 cells in our study)—did not make anti-MPG responses. The finding that the cell-bound MPG antigen cannot induce anti-MPG responses follows, as had been reported earlier (23).

Furthermore, we have demonstrated that, similar to Ab1, Ab3 is biologically active, as demonstrated by the ability to inhibit melanoma cell invasion in the *in vitro* Matrigel assay. The ability of *in vitro* assays for invasion of Matrigel basement membrane matrix to predict invasive behavior *in vivo* has been demonstrated by numerous groups (21, 24, 25). Of the various *in vitro* assays for tumor cell invasion, it appears to suffer least from intralaboratory variations and to be most versatile in its applications (25). To our knowledge, it has not been reported previously that an anti-Id can induce a functionally active anti-tumor antibody response in non-human primates. However, it should be noted that our approach does not show directly that the Ab3 or MEM136 will inhibit the spreading of tumor or metastasis *in vivo*. In addition, in the Matrigel assay, the Ab3-mediated inhibition of melanoma cell invasion was found to be less pronounced relative to the Ab1 when tested at the same concentration. This apparent

Table 1. Binding of anti-Id mAb I-Mel-2 induced anti-anti-Id antibodies to melanoma cells

^{125}I-labeled antibody*	Specific activity, cpm/mg	Antibody added		Antibody bound		Specifically bound cpm‡	% bound antibody
		cpm	ng of antibody	cpm†	ng of antibody		
I-Mel-2-Ab3	9.8 × 10^6	6,472	0.656	840 ± 58	0.08	338	4.5
		10,362	1.05	1422 ± 74	0.14	863	8.2
		23,298	2.36	2270 ± 31	0.23	1421	6.1
5A8-Ab3	4.7 × 10^6	6,366	1.34	502 ± 14	0.1		
		10,170	2.14	559 ± 6	0.12		
		24,108	5.08	849 ± 105	0.18		

Various amounts of ^{125}I-labeled anti-anti-Id antibodies were incubated with Colo38 cells (2 × 10^5) for 1 hr at 4°C. Cells were then washed six times with phosphate-buffered saline/bovine serum albumin/NaN_3 and bound radioactivity was measured in a γ counter.

*Anti-anti-Id (Ab3) antibodies were purified from serum from monkeys immunized with mAb I-Mel-2 or 5A8 in SAFm and labeled with ^{125}I.

†Mean ± SEM.

‡Determined by subtracting cpm bound to Colo38 cells incubated with ^{125}I-labeled 5A8-Ab3 antibodies from cpm bound to Colo38 cells incubated with ^{125}I-labeled I-Mel-2-Ab3 antibodies.

Table 2. Effect of MEM136 (Ab1) and I-Mel-2-induced Ab3 on basement membrane matrix invasion by melanoma cells

Antibody	Cell line	Cellular invasion of basement membrane matrix*		
		% of control	Range	$P^{\dagger}$
5A8-Ab3	Colo38	95.0		NS
mAb 05/13	Colo38	133.0		NS
MOPC21	Colo38	130.0		NS
MEM136	Colo38	2.6	3.0–2.3	0.001
I-Mel-2-Ab3	Colo38	57.0	75–25	0.07
MOPC21	Meljur	110		NS
MEM136	Meljur	9.8	12.0–7.4	0.001
I-Mel-2-Ab3	Meljur	50.0	61–39	0.001
MEM136	MeWo	96.0		NS
I-Mel-2-Ab3	MeWo	104		NS

Colo38 and Meljur cells are MPG-positive; MeWo cells are MPG-negative. Data are averages of four to eight replicates for each point. All antibodies were present at 30 μg/ml except for mAb 05/13, which was used as a neat culture supernatant.

*A 1:20 dilution of Matrigel (Collaborative Research) in serum-free medium.

†NS, no statistical difference between data sets. Statistical analysis was carried out using the Mann–Whitney U two-tailed test.

discrepancy may be explained by the following. (*i*) It could be a reflection of the concentration of Ab1′ in the polyclonal Ab3 population (4–8% of the Ab3 bound to Colo38 cells specifically). (*ii*) A lesser effect of polyclonal Ab3 on melanoma cell invasion could be due to the fact that only a small fraction of the Ab3 mimics MEM136 specificity (≈1% of the Ab3 population competed with ^{125}I-labeled MEM136 to bind to Colo38 cells). (*iii*) The difference could be ascribed to variations in the affinity of MEM136 or Ab3 toward the MPG epitope. This argument could be used to explain the lack of ability of the Ab3 to inhibit the binding of MEM136 to melanoma cells, as these antibodies have a lower affinity for the MPG antigen than the Ab1. This possibility appears unlikely, however, since ^{125}I-labeled anti-anti-Id binding to melanoma cells could not be inhibited by MEM136 (data not shown).

How I-Mel-2 induces an anti-MPG response with a specificity different from MEM136 is unclear. Earlier studies in rodents indicate that anti-Id mAb have the ability to induce antigen-specific humoral responses having different epitope specificities than the original Ab1 (19, 26–28). As suggested recently (29), I-Mel-2 could be termed a "network antigen" instead of an "internal image" antibody to describe its functional attribute. Finally, a role of T helper cells for the induction of anti-MPG responses by I-Mel-2 is believed to be important. The I-Mel-2 may provide the MPG epitope on a sufficiently foreign protein to allow cognate T help.

Our data imply that an anti-idiotypic antibody may overcome unresponsiveness to a cell-bound MPG antigen and induce a biologically significant antibody response against an epitope that is present on normal tissue. We cannot say with complete certainty that an idiotype system (i.e., I-Mel-2) is the only way to break tolerance to MPG in primates, as the definitive experiment—namely, use of biochemically purified antigen in immunization studies—has not yet been carried out. The problems of obtaining sufficient quantities of refined, cell-free MPG for such experiments are substantial hurdles, but, nevertheless, until these trials have been conducted we will be cautious in our claims for the ability of I-Mel-2 to break immunal unresponsiveness.

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