Preclinical Evaluation in Nonhuman Primates of Murine Monoclonal Anti-Idiotype Antibody that Mimics the Disialoganglioside GD2

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ABSTRACT

The antiganglioside GD2 monoclonal antibody 14G2a (Ab1) served as an immunogen to generate the anti-idiotype (anti-Id) 1A7 (IgG1, κ), which mimics GD2 both antigenically and biologically. Anti-Id 1A7 induced anti-GD2 antibodies in mice and rabbits. In this preclinical study, a pair of cynomolgus monkeys, immunized with 1A7 that had been mixed with QS-21 adjuvant, produced anti-anti-idiotype antibodies (Ab3), which reacted with the GD2-positive melanoma cell line M21/P6 cells but not with GD2-negative LS174-T cells. The Ab3 shared Ids with mAb 14G2a (Ab1), as demonstrated by their ability to inhibit binding of 1A7 to this Ab1. The Ab3 bound specifically to purified GD2 antigen and competed with the Ab1 14G2a in binding to a GD2-positive melanoma cell line or to purified GD2, suggesting that Ab1 and Ab3 may bind to the same epitope and may behave as an Ab1-like antibody (Ab1'). The isotype of the GD2-specific antibodies was mostly IgG in nature. The specificity of the antibodies for GD2 was further confirmed by dot blot analysis. These antisera also specifically lysed GD2-positive target cells in an antibody-dependent cellular cytotoxicity assay. The induction of anti-GD2 responses in monkeys did not cause any apparent side effects, despite the fact that GD2 antigen is expressed by many normal tissues of these animals. Taken together, these results suggest that anti-Id 1A7 can induce GD2-specific antibodies in nonhuman primates and can thus serve as a potential network antigen for triggering active anti-GD2 antibodies in patients with GD2-positive neuroectodermal tumors.

INTRODUCTION

Tumors of neuroectodermal origin, such as melanomas and neuroblastomas, have a poor clinical prognosis. Neuroblastoma is the major cause of morbidity and mortality among children bearing solid tumors. The incidence of malignant melanoma is increasing worldwide. One of the changes that occur in the process of malignant transformation is an altered pattern of cell surface ganglioside expression (1). In malignant melanoma, activation of glycosylating enzymes leads to increased expression of GD2, GD3, GM2, and 9-O-acetyl GD3 (2, 3). All of these can be considered potential targets for treatment with mAbs and vaccines (4). The ganglioside GD2 is highly expressed on cells of neuroectodermal origin, including melanoma, neuroblastoma, and small cell carcinoma of the lung (5–7). In humans, GD2 is only weakly immunogenic and generally induces T-cell-independent humoral immune responses (8).

Several mouse mAbs against GD2 have shown antitumor effects in preclinical and clinical studies (9–14). However, there are several drawbacks in using murine mAb for passive immunotherapy in humans, including the development of human antimouse immunoglobulin antibody responses that prevent further therapy. Human-mouse chimeric anti-GD2 antibody ch14.18 induced remissions in patients with metastatic neuroblastoma (15) and showed encouraging results in patients with malignant melanoma (16). The mechanisms believed to be involved in the ability of anti-GD2 mAb to suppress the growth of tumors in vivo are ADCC and/or complement-mediated cytotoxicity. To increase the therapeutic efficacy, clinical trials were initiated combining therapy with interleukin 2 and either the murine 14G2a antibody or the ch14.18 antibody (17, 18). Also, in vitro studies have shown that mAbs against gangliosides potentiate lymphocyte response to various stimuli. Therefore, an additional benefit for the therapeutic application of antibodies against GD2 may be the potentiation of an immune response to tumor cells. In addition, GD2 appears to play a role in melanoma cell adhesion to solid substrates, and antibodies against GD2 inhibit melanoma cell attachment, a process that is critical for metastasis (19, 20). For these reasons, there has been considerable interest in trying to induce an active immune response against GD2 in GD2-positive cancer patients.

Because cancer patients are often immunosuppressed and GD2 is poorly immunogenic, triggering an active immune re-
spontaneous response to such antigens represents a challenge for cancer therapy. One approach to this problem has been to use internal anti-idiotype mimicking immunity. We reported previously in Refs. 21 and 22.

To explore the potential of anti-Id as a vaccine, we have produced one mouse mAb anti-Id 1A7 against 14G2a, which is a murine anti-GD2 mAb of IgG2a isotype. We reported that 1A7 induced specific humoral responses in mice and rabbits. In an effort to create a more relevant clinical model, we used cynomolgus monkeys (Macaca fascicularis) to investigate the effect of the murine anti-Id mAb 1A7, which mimics antigenic determinants on GD2 ganglioside, in these animals. Preclinical studies made use of the same type of adjuvant (QS-21) and 1A7 had been used subsequently in clinical trials. Because the normal tissue distribution patterns of GD2 in monkeys and humans are quite similar, these studies will likely predict the safety and efficacy of this anti-idiotype in inducing antitumor antibodies in melanoma and other GD2-positive cancer patients.

MATERIALS AND METHODS

Cell Lines. The human melanoma cell line M21/P6 and the human colorectal cancer-derived colon cancer cell line LS174-T were grown in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 units/ml of penicillin (Life Technologies, Inc.), and 100 μg/ml streptomycin (Life Technologies, Inc.). M21/P6 cells, which express GD2 on their surfaces, were used as positive controls and LS174-T cells, which lack this antigen, served as negative controls.

Antibodies. The Ab1 mAb 14G2a (IgG2a, κ), which recognizes a GD2 epitope, was used to immunize syngeneic BALB/c mice for the production of anti-Id mAb 1A7 (IgG1, κ). 1A7 is specific for an idiotope of 14G2a and behaves as an internal image of GD2. The mAb 3H1 (IgG1, κ) is a murine anti-Id mAb that mimics the human carcinoembryonic antigen (21) and was used as a control.

Adjuvant. An adjuvant is generally required to augment the immunogenicity of the anti-Id vaccine. QS-21, a saponin extract from the bark of the South American soap bark tree (Quillaja saponaria monila), which has significant adjuvant activity (23-25), could be used on a trial basis because it is approved by the United States Food and Drug Administration for use in humans in experimental vaccine therapy. Therefore, we immunized monkeys in this preclinical study with 14G2a and whether the 1A7 was a true anti-Id, monkey sera were checked for their ability to inhibit the binding of 125I-labeled 1A7 to 14G2a (Ab1) bound to microtiter plates or vice versa. An unrelated Ab1-Ab2 system (mAb 8019-3H1) was used as control.

Inhibition of Binding of Ab1 to a GD2-positive Cell Line by Ab3. To ascertain whether Ab3 in monkey sera shared idiotopes with mAb 14G2a (Ab1) and whether the Ab3 was an anti-Id, monkey sera were checked for their ability to inhibit the binding of 125I-labeled 1A7 to 14G2a (Ab1) bound to microtiter plates or microtiter plates or vice versa. Unrelated Ab1-Ab2 system (mAb 8019-3H1) was used as control.

Inhibition of Binding of Ab1 to a GD2-positive Cell Line by Ab3. To determine whether the Ab3 would be able to compete with Ab1 for binding to GD2-positive human melanoma cell line, binding of radiolabeled mAb 14G2a to M21/P6 cells was tested for inhibition in the presence of different dilutions of immunized sera, purified Ab3 or Ab1. Percentage of inhibition measured in these assays was calculated according to the formula described earlier (27).

Binding of Ab3 to Purified GD2. Anti-GD2 reactivity was measured in the sera of monkeys or purified Ab3 with different gangliosides: GM1 [Galβ1→3GalNAcβ1→4NeuAcα2→3Galβ1→4Glc→cer], GM2 [GalNAcβ1→4NeuAcα2→3Galβ1→4Glc→cer], GM3 [NeuAcα2→3Galβ1→4Glc→cer], GD3 [NeuAcα2→8NeuAcα2→3Galβ1→4Glc→cer], GD2 [GalNAcβ1→4 NeuAcα2→3Galβ1→4Glc→cer], and GT1b [NeuAcα2→3Galβ1→3GalNAcβ1→4 NeuAcα2→8 NeuAcα2→3 Galβ1→4Glc→cer], which were adsorbed onto 96-well microtiter plates (250 ng/well). After blocking with 1% BSA in PBS, test samples and Ab1 were added to plates at different dilutions and incubated for 4 h at RT with shaking. After washing, the bound antibodies were detected with alkaline phosphatase conjugated goat antihuman immunoglobulin serving as second antibody. Isotype of the antibodies was determined using

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goat antihuman IgG (γ chain specific) and goat antihuman IgM (μ chain specific) as second antibodies.

**Flow Cytometric Analysis with Ab1 and Ab3.** GD2-positive melanoma cells M21/P6 (5 × 10^5 per tube) and GD2-negative colorectal cancer-derived LS174-T cells (5 × 10^5 per tube) were reacted with Ab1 (14G2a) or Ab3 sera at 1:100 dilution at 4°C for 2 h. After washing, the cells were incubated with either FITC-labeled goat antihuman immunoglobulin or goat antimouse immunoglobulin (for Ab1) labeled antibody (Tago Immunochemicals) for 30 min at 4°C. The cells were washed with cold PBS, fixed in 3% paraformaldehyde, and analyzed by flow cytometry (FACStar, Becton Dickinson, San Jose, CA).

**Dot Blot Analysis.** Reactivity of immunized sera and purified Ab3 for the presence of antiganglioside antibodies against various gangliosides was also measured by immunoblotting. Purified gangliosides (2 μg each of GM3, GM2, GM1, GD3, GD2, and GT1b) were spotted on strips of polyvinylidene difluoride cellulose membrane at 1-cm intervals. After blocking with 3% BSA in PBS, the strips were incubated with purified Ab3 or Ab1 (10 μg/ml) for 4 h at RT. After washing, the strips were incubated with alkaline phosphatase conjugated goat anti-

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**Table 1 Analysis of monkey anti-anti-Id sera generated with anti-Id mAb 1A7**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Plate coated with</th>
<th>125I-labeled anti-Id</th>
<th>Sample</th>
<th>Ab3 sera</th>
<th>Ab3 sera (control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sandwich RIA*</td>
<td>1A7 (Ab2)</td>
<td>1A7</td>
<td>PRO 685</td>
<td>47637</td>
<td>48603</td>
</tr>
<tr>
<td></td>
<td>3H1 (unrelated Ab2)</td>
<td>1A7</td>
<td>PRO 778</td>
<td>167</td>
<td>447</td>
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<tr>
<td></td>
<td>1A7 (Ab2)</td>
<td>3H1</td>
<td>Preimmune sera</td>
<td>525</td>
<td>478</td>
</tr>
<tr>
<td>Inhibitionb</td>
<td>14G2a (Ab1)</td>
<td>1A7</td>
<td>PRO 723</td>
<td>566</td>
<td>478</td>
</tr>
<tr>
<td></td>
<td>1A7 (Ab2)</td>
<td>14G2a</td>
<td>PRO 872</td>
<td>83</td>
<td>6</td>
</tr>
</tbody>
</table>

* Ab3 sera obtained after fourth immunization were diluted with PBS containing normal mouse immunoglobulin (500 μg/ml). The sera were preincubated with normal mouse Ig prior to the assay. Ab3 at a dilution of 1:40 was incubated with anti-Id mAb 1A7 or 3H1 (250 ng/well) coated on the microtiter plate and then reacted with 125I-labeled 1A7 or 3H1 (90,000 cpm) by a sandwich assay. The results are expressed as bound cpm in a sandwich assay. The results are presented as mean cpm (n = 3). The SD of the data was <10%. Preimmune sera were used from monkey PRO 778 collected before immunization.

b For the binding inhibition assay between Ab2 and Ab1, purified Ab1 14G2a was used to coat the plate (500 ng/well), and the binding of radiolabeled Ab2 to Ab1 was tested in the presence of different dilutions of Ab3 sera. For the binding inhibition assay between Ab1 and Ab2, purified Ab2 1A7 was used to coat the plate (500 ng/well), and the binding of radiolabeled Ab1 to Ab2 was tested in the presence of different dilutions of Ab3 sera. The sera were preincubated with normal mouse Ig prior to the assay. The results are expressed as percentage of inhibition at a dilution of 1:40.

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**Fig. 1** Inhibition of Ab1 (14G2a) binding to GD2-positive M21/P6 melanoma cells by monkey Ab3 sera. M21/P6 (5 × 10^5) cells were incubated with different dilutions of immune monkey sera and a fixed amount of radiolabeled 14G2a (90,000 cpm). Percentage of inhibition was calculated and plotted against serum dilution. Monkeys [PRO 778 (□) and PRO 685 (□)] were immunized with 1A7 and QS-21, whereas monkey PRO 872 (△) was immunized with control 3H1 and QS-21. ○, preimmune. Data points, mean of three experiments; bars, SD. Inset, inhibition curve using cold 14G2a.

**Fig. 2** Isotype analysis of sera (1:100 dilution) from monkeys immunized with anti-Ids 1A7 or 3H1. Anti-GD2 reactivity was measured by ELISA (see “Materials and Methods”); columns, mean absorbance at 405 nm. Monkeys [PRO 778 (■) and PRO 685 (□)] were immunized with 1A7 and QS-21, whereas monkey PRO 872 (△) was immunized with control Ab2 3H1 and QS-21. ■, preimmune.
Fig. 3 Immune flow cytometry of M21/P6 cells with monkey Ab3 sera. A. Tumor cells reacted with preimmune sera and Ab3 sera (1:100 dilution) from monkey (PRO 778) immunized with 1A7 and QS-21. B. Tumor cells reacted with PBS control and 14G2a (Ab1). The fluorescent intensity obtained with normal mouse IgG2a as control was 1.64%, which was comparable to PBS control (data not shown). C. GD2-negative LS174-T control cells reacted with preimmune serum and Ab3 sera obtained from monkey (PRO 778) immunized with 1A7 + QS-21. Bound antibody was detected by flow cytometry after incubation with FITC-labeled second antibody (see “Materials and Methods”).

Fig. 4 ADCC. 

\[
\% \text{ specific lysis} = \frac{\text{Experimental lysis} - \text{spontaneous lysis}}{\text{Maximum lysis} - \text{spontaneous lysis}} \times 100
\]

Immunoperoxidase Staining of Monkey Tissues with Ab1 and Ab3. The reactivities of Ab1 and purified Ab3 at a concentration of 50 µg/ml were compared on fixed specimens of monkey brain and peripheral nerve tissues by a very sensitive staining method (biotin-streptavidin reagents; Vector, Burlingame, CA) as described in detail elsewhere (26). All sections were counterstained with Meyer’s hematoxylin. Pertinent specificity tests were performed, including block of the endogenous peroxidase, omission of the first layer, or substitution of non-immune homologous serum for the specific antiserum and P3-653 myeloma culture supernatant as the control.

RESULTS

Induction of Anti-anti-Id (Ab3) Responses in Monkeys.

The sera from monkeys were obtained 10 days after the fourth immunization and analyzed for Ab3 responses by sandwich RIA and inhibition of Ab2 binding to Ab1 (Table 1). For these assays, the sera were pretreated with normal mouse immunoglobulin (500 µg/ml) to block anti-isotypic and anti-idiotypic reactivities. Ab3 sera from monkeys (PRO 778 and PRO 685) at a concentration of 50 µg/ml were compared on fixed specimens of monkey brain and peripheral nerve tissues by a very sensitive staining method (biotin-streptavidin reagents; Vector, Burlingame, CA) as described in detail elsewhere (26). All sections were counterstained with Meyer’s hematoxylin. Pertinent specificity tests were performed, including block of the endogenous peroxidase, omission of the first layer, or substitution of non-immune homologous serum for the specific antiserum and P3-653 myeloma culture supernatant as the control.

ADCC. M21/P6 cells were labeled in 1 ml of complete DMEM containing 200 µCi of \(^{51}\text{Cr}\) by incubation for 1 h at 37°C. The cells were then washed with DMEM without FCS and suspended in the same medium. Cells (1 × 10^4 in 25 µl) were added to individual wells of a 96-well microtiter plate together with different dilutions of crude immune serum. Peripheral blood mononuclear cells, isolated by Ficoll/Hypaque gradient centrifugation from normal human donors, were then added to the plate at the E:T ratio of 50:1. The plates were centrifuged at 400 × g for 2 min and incubated 4 h at 37°C in a humidified atmosphere containing 5% CO₂. After incubation, the plates were centrifuged at 400 × g for 5 min, and cpm released in the supernatant were measured. The amount of spontaneously released \(^{51}\text{Cr}\) was also measured in wells that contained only labeled target cells. Total released cpm was assessed by lysing labeled cells with 1% NP40. The percentage of lysis was calculated by the formula given below. To calculate lysis specifically attributable to ADCC, the percentage of lysis due to effector cells in the absence of antibody (i.e., natural killer cells), was subtracted from each value obtained above.
Inhibition of Abi (14G2a, U) binding to purified GD2 by purified Ab3 or Ab1. A GD2-coated microtiter plate was incubated with different dilutions of purified Ab3 or Ab1 and a fixed amount of radiolabeled 14G2a (9 × 10^6 cpm). Monkeys (PRO 778 (□) and PRO 685 (■)) were immunized with 1A7 and QS-21.

Fig. 5 Anti-GD2 specificity of purified monkey Ab3. Purified Ab1 or Ab3 (10 μg/ml) was reacted with different gangliosides on polyvinylidene difluoride membrane. Bound antibody was detected by using alkaline phosphatase conjugated goat antihuman immunoglobulin or goat antimouse immunoglobulin (for Ab1) as second antibody. Lane 1, sample was incubated with Ab1 (14G2a); Lanes 2 and 3, samples were reacted with purified Ab3 from monkey sera (PRO 778 and PRO 685) immunized with 1A7 and QS-21; Lane 4, sample was incubated with control Ab3 from monkey immunized with 3H1 and QS-21.

1A7 and bound only minimally to unrelated Ab2 (3H1). Monkey Ab3 sera also inhibited the binding of radiolabeled Ab2 to Ab1 by 75 and 83%, even at a dilution of 1:40 (Table 1). There was no inhibition with preimmune sera or sera obtained from monkeys (PRO 872 and PRO 723) immunized with the unrelated Ab2 3H1. Moreover, sera from monkeys immunized with 1A7 (PRO 778 and PRO 685) inhibited the binding of radiolabeled Ab1 (14G2a) to Ab2 (1A7) by more than 80% at a serum dilution of 1:40, whereas preimmune sera and sera from monkeys immunized with unrelated Ab2 (3H1) showed minimal inhibition. These results indicate that monkey Ab3 sera share Ids with Ab1.

Induction of Antitumor Cell Antibody Response. To determine whether sera from monkeys immunized with 1A7 bound specifically to GD2-positive human melanoma cells, the binding of 125I-labeled Ab1 to the M21/P6 cells was tested for inhibition in the presence of different dilutions of monkey Ab3 sera. As shown in Fig. 1, Ab3 sera obtained after the fourth immunization inhibited the binding of mAb 14G2a to M21/P6 cells at different dilutions, whereas preimmune sera and control monkey sera showed minimal inhibition. Specific binding of Ab3 sera to purified GD2-coated microtiter plates was also tested by ELISA. As shown in Fig. 2, sera of monkeys immunized with 1A7 (PRO 778 and PRO 685) contained primarily IgG antibodies with a minimum amount of IgM antibodies directed against GD2, and these sera did not react with other gangliosides, such as GD3 and GM2. Preimmune sera and sera from monkeys immunized with 3H1 did not show appreciable binding to GD2.

To determine the reactivity of monkey Ab3 sera with cell surface GD2, M21/P6 cells were tested by immune flow cytometry. As shown in Fig. 3, Ab3 sera from immunized monkeys showed distinct binding (Fig. 3A) that was similar to the binding pattern obtained with the Ab1 14G2a (Fig. 3B). Significant binding was not obtained with LS174-T cells, which do not express GD2 (Fig. 3C). We repeated this experiment with goat antihuman F(ab')2-IgG (γ chain specific) and goat antihuman F(ab')2-IgM (μ chain specific) FITC-labeled second antibody. The sera from monkeys immunized with 1A7 did not show any distinct binding to M21/P6 cells when goat antihuman F(ab')2-IgG-FITC-labeled antibody was used as the second antibody (data not shown). These results further confirm that sera from monkeys immunized with 1A7 mainly produced GD2-specific antibodies of the IgG but not the IgM isotype.

Mediation of ADCC of M21/P6 Target Cells by Ab3. To assess whether sera from monkeys immunized with 1A7 could induce ADCC in conjunction with normal human peripheral blood mononuclear cells serving as effector cells, M21/P6 cells were labeled with 51Cr and incubated in the presence of sera from immunized monkeys and effector cells. As depicted in Fig. 4, the monkey sera induced specific lysis in 40–50% of target cells at a serum dilution of 1:10 and E:T cell ratio of 50:1; however, preimmune sera and sera from monkeys immunized with unrelated Ab2 3H1 produced only 4–8% specific lysis. Insignificant lysis due to natural killer cells was subtracted from each value obtained. Unrelated cell lines were not used as controls in this assay.

Reactivity of Purified Ab3 from Monkey Sera. The Ab3 antibodies were purified from sera as described in "Mate-
Fig. 7  Immunoperoxidase staining of monkey tissues with PBS-BSA, Ab1, and Ab3. Hippocampus of brain of monkey PRO 778 treated with PBS-BSA (A), Ab1 14G2a (B), and purified Ab3 of monkey PRO 778 (C); right sural nerve of monkey PRO 778 treated with PBS-BSA (D), Ab1 14G2a (E), and purified Ab3 of monkey PRO 778 (F); cortex of brain of monkey PRO 685 treated with PBS-BSA (G), Ab1 14G2a (H), and purified Ab3 of monkey PRO 685 (I). A, C, D, F, G, and I were treated with antihuman immunoglobulin biotinylated reagent as a second antibody, whereas B, E, and H were treated with antimouse immunoglobulin biotinylated reagent as second antibody.

The specificity of purified Ab3 for GD2 was further confirmed by dot blot analysis (Fig. 5). As described in “Materials and Methods,” individual gangliosides (2 μg) were spotted onto the strips and incubated with different antibodies. In Fig. 5, Lanes 2 and 3, samples were incubated with Ab3 obtained from monkeys immunized with 1A7 (PRO 778 and PRO 685), and the sample in Lane 1 was incubated with Ab1 14G2a. Ab3 recognized GD2 with a pattern identical to Ab1 and did not recognize other gangliosides. The sample in Lane 4 was treated with unrelated Ab3 (monkey immunized with control Ab2 3H1) and showed no reactivity with GD2.
Competition of Murine Ab1 and Monkey Ab3 for Binding to Purified Ganglioside GD2. If Ab3 has a binding site similar to that of Ab1, it should compete with Ab1 for binding to purified GD2. Thus, a fixed amount of radiolabeled Ab1 was coincubated with different amounts of purified Ab3 or Ab1 or control Ab3 preparations on GD2-coated microtiter plates (Fig. 6). Purified Ab3 from monkey PRO 778 at a concentration of 75 ng inhibited the binding of Ab1 to GD2 by 65%, whereas Ab3 from monkey PRO 685 inhibited it by 30%. These results indicate that Ab2-immune monkey antibody binds to the same epitope as Ab1. Therefore, the Ab3 preparations contain antibody molecules with Ab1' properties.

Toxicity Studies. The induction of Ab3 responses in monkeys did not cause any apparent side effects in these animals despite the presence of GD2 in some normal tissues as determined by immunoperoxidase staining with mAb l4G2a. Only mild local swelling and irritation were observed at the injection site as a result of multiple immunizations. The monkeys were routinely checked by physical examinations and weight measurement. They did not show any abnormalities in these regards, including neurological problems. All monkeys received a total of five immunizations. Seven months after immunization, they were sacrificed, and a detailed autopsy was performed; the autopsy did not indicate any abnormalities in any of the organs by histopathology. The monkey tissues were fixed by immunohistochemical staining for the presence of Ab3 bound to those tissue specimens that express GD2 in humans (i.e., neuroectodermal tissues). The results of the immunohistochemical evaluation suggest that there was no in vivo Ab3 binding to monkey peripheral nerve or brain tissues (Fig. 7, A, D, and G). Treatment of the same tissue sections in vitro with excess mAb l4G2a or purified Ab3 showed distinct binding suggesting the presence of GD2 in these tissues (Fig. 7, B, E, H, C, F, and I). Overall, the immunohisto pathological studies suggest that there were no toxicities induced by the anti-Id 1A7 and QS-21 vaccine treatment.

DISCUSSION

This study was conducted in a more clinically relevant model than mice or rabbits to evaluate safety and efficacy of a murine anti-Id vaccine to be administered to melanoma patients for generation of antitumor immune responses. In this preclinical study, we used QS-21 as an adjuvant. In a recent report, QS-21 was described as an effective adjuvant when used with a GM2-keyhole limpet hemocyanin-conjugated vaccine (28). Moreover, the same clinical study also revealed that QS-21 induced a consistent IgG antibody response in melanoma patients. The results described here demonstrate that a murine anti-Id mAb, 1A7, raised against a GD2-specific mAb 14G2a, can induce a specific anti-GD2 humoral response in cynomolgus monkeys. The anti-anti-Id antibody (Ab3) concentration was quite high; 2.6 mg of purified Ab3 was recovered from 10 ml of an immune monkey serum (260 μg/ml serum), and slightly less from the other monkey. The Ab3 response induced by 1A7 in monkeys has been extensively characterized. They inhibited the binding of Ab1 to the GD2-positive melanoma cell line M21/P6. Flow cytometry and dot blot analysis further confirmed the antigenic specificity of Ab3. Although we could not quantitate the amount of true anti-GD2 antibodies in Ab3 serum, competitive binding inhibition studies performed with purified Ab1 and Ab3 (Fig. 6) suggest that Ab3 response elicited by 1A7 was primarily Ab1' in nature. This antibody response was found to be mainly of IgG isotype. To quantitate the amount of true anti-GD2 antibodies in Ab3 serum, we needed to use GD2 as an affinity matrix to purify anti-GD2 antibodies. Unfortunately, we do not have enough purified GD2 and it is not available commercially.

Although GD2 is present in some normal tissues in monkeys, no detectable side effects were observed except local swelling and irritation at the injection site, due to multiple immunizations. Seven months after the completion of immunizations, autopsies were performed on all monkeys. The autopsy evaluations of different organs indicated no abnormalities induced by the 1A7 vaccinations.

Due to economic reasons, we used only two monkeys (1 male and 1 female) in each group treated with a single dose of 2 mg of anti-Id vaccine. However, both monkeys showed excellent humoral and cellular immune responses. We also had an appropriate control, a pair of monkeys (one male and one female) immunized with the unrelated Ab2 (3H1). This particular study has direct clinical relevance because normal tissue expression of GD2; therefore, immunological tolerance to GD2 should be very similar in monkeys and humans. Studies by Livingston (10) of the Memorial Sloan-Kettering Institute have indicated that melanoma patients in general do not mount any antibody response to GD2. A variable degree of immunological tolerance exists with all natural gangliosides in proportion to their expression on normal tissues (10). Another recent report on the induction of antitumor antigen immunity in nonhuman primates also indicated that an appropriate anti-Id could break unresponsiveness to a human melanoma-associated proteoglycan antigen (29). The study described here provides the critical preclinical data to justify clinical trial with this anti-Id vaccine. Other investigators have also used anti-Ids to induce responses against different gangliosides in preclinical and clinical studies (9, 30-33). Recently, the investigators at the Memorial Sloan-Kettering Institute have used an anti-Id, designated BEC2, which mimics GD3, to generate GD3-specific antibodies in melanoma patients (34). Encouraging results have been obtained that suggest the utility of anti-Id immunization as a surrogate for ganglioside vaccination. Mittelman et al. (35) used an anti-id mAb MK 2-23 that mimics the human high molecular weight melanoma-associated antigen to induce anti-high molecular weight melanoma-associated antigen antibodies in melanoma patients (35). Recently, we have obtained an Investigational New Drug authorization from the United States Food and Drug Administration for 1A7. The potential role of this anti-Id vaccine to induce humoral immune responses in patients with GD2-positive cancer can thus be investigated directly in a clinical trial.

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