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

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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-
Anti-idiotype Mimicking GD2 Ganglioside in Monkey

spose to such antigens represents a challenge for cancer therapy. One approach to this problem has been to use internal image Ab2s as antigen substitutes to induce antitumor immunity (reviewed in Refs. 21 and 22).

To explore the potential of anti-Id as antitumor 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. Here, 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. This preclinical study made use of the same type of adjuvant (QS-21) and the same dosage of 1A7 that will be 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 monilia), 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 Ab2 1A7 and QS-21. The 100-µg dose of QS-21 was selected based on studies by other investigators (reviewed in Ref. 10). Cambridge Biotech (Cambridge, MA) made this adjuvant available to us.

Immunization of Monkeys. Cynomolgus monkeys (two per group; weight, 2–4 kg) were immunized with s.c. injections of 2 mg of purified Ab2 1A7 or control anti-Id 3H1 (specific for carcinoembryonic antigen) mixed with 100 µg of QS-21 as adjuvant. Monkeys housed at the White Sands Research

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 a true anti-anti-Id, monkey sera were tested 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 Assay. To ascertain whether Ab3 in monkey sera shared idiotopes with mAb 14G2a (Ab1) and whether the Ab3 was a true anti-anti-Id, monkey sera were tested 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 determine whether crude Ab3 sera or purified Ab3 is 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→4(NeuAcα2→3)Galβ1→4Glc→cer], GM2 [GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glc→cer], GM3 [NeuAcα2→3Galβ1→4Glc→cer], GD3 [NeuAcα2→8NeuAcα2→3Galβ1→4Glc→cer], GD2 [GalNAcβ1→4(NeuAcα2→8NeuAcα2→3)Galβ1→4Glc→cer], and GT1b [NeuAcα2→3Galβ1→3GalNAcβ1→4(NeuAcα2→8NeuAcα2→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

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 x 10⁵ per tube) and GD2-negative colorectal cancer-derived LS174-T cells (5 x 10⁵ 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-
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. M21/P6 cells (1 x 10^4) were incubated with 100 μl of different monkey sera (1:10 dilution) using human peripheral blood lymphocytes as effector cells at a ratio of 50:1 for 4 h at 37°C. Radioactivity released in the supernatant was measured, and specific lysis was calculated according to the formula as described in "Materials and Methods." Monkeys PRO 778 and PRO 685 were immunized with 1A7 and QS-21, whereas monkey PRO 872 was immunized with 3H1 and QS-21.

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 antiallotypic 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.

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Fig. 6 Inhibition of Ab1 (14G2a, ■) 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.

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Inhibition of Abi (l4G2a, U) binding to purified GD2 by purified Ab3 or AbI. A GD2-coated microtiter plate was incubated with different dilutions of purified Ab3 or AbI and a fixed amount of radiolabeled 14G2a (9 × 10^6 cpm). Monkeys IPRO 778 (D and PRO 685) were immunized with 1A7 and QS-21.

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The Figure 6 graph shows the inhibition of Ab1 (14G2a) binding to GD2 when incubated with purified Ab3 or Ab1. Different dilutions of these antibodies were tested, with Ab1 showing a significant decrease in binding at lower concentrations while Ab3 showed a more gradual decrease. The figure illustrates the effectiveness of Ab1 and Ab3 in inhibiting GD2 binding, with Ab1 being more potent at lower concentrations.
Materials and Methods,” and their reactivity against different gangliosides was studied by ELISA. Immune monkey Ab3 reacted specifically with GD2 coated onto microtiter plates, whereas only background reactivity was detected with other gangliosides (data not shown).

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.

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.
**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 cynomolgous 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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