Antibody Responses in Melanoma Patients Immunized with an Anti-Idiotype Antibody Mimicking Disialoganglioside GD2

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ABSTRACT

We initiated a clinical trial for patients with advanced malignant melanoma treated with an anti-idiotype antibody that mimics the disialoganglioside GD2. We report the clinical and immune responses of the first 12 patients entered into this trial. Patients received 1-, 2-, 4-, or 8-mg doses of the anti-idiotype antibody mixed with 100 μg of QS-21 adjuvant every other week, four times, and then monthly. Twelve patients have been on trial for 2–23 months, and all of them have generated immune responses. Patients were removed from the study if they demonstrated disease progression. Hyperimmune sera from all 12 patients revealed an anti-anti-idiotypic Ab3 response, as demonstrated by the inhibition of Ab2 binding to Ab1 by patients’ immune sera. To further test the anti-anti-idiotypic response, patients’ Ab3 antibodies were affinity purified on Sepharose 4B columns containing adsorbed immunizing anti-idiotype immunoglobulin. Purified Ab3 of all patients studied inhibited binding of Ab1 to a GD2-positive cell line. Purified Ab3 also inhibited binding of Ab1 to purified GD2, in a manner comparable to equal quantities of purified Ab1. The patient Ab3 was truly an Ab1’ because it specifically bound to purified disialoganglioside GD2. The idiotypic specificity of the Ab3 antibody was predominantly IgG, with only minimal IgM. The predominant IgG subclass was IgG1, with approximately equal quantities of IgG2, IgG3, and IgG4. These Ab3 antibodies reacted specifically with tumor cells expressing GD2 by immune flow cytometry and immunoperoxidase assays. Five patients’ Ab3 antibodies studied for antibody-dependent cellular cytotoxicity were positive. One patient had a complete clinical response, with resolution of soft tissue disease, and six patients had stable disease, ranging from 9 to 23 months, and are being continued on vaccine therapy. Toxicity consisted of local reaction at the site of the injection, including induration and pain that generally resolved within a few days. Mild fever and chills were observed in 75% of the patients but rarely required acetaminophen. There was no additional toxicity, including abdominal pain that was previously seen with infusion of murine monoclonal anti-GD2 antibody. Current trials include patients with stage III melanoma and small cell lung cancer. Future trials will attempt to enhance the antitumor response by the addition of interleukin 2, granulocyte macrophage colony-stimulating factor, and other cytokines, together with the 1A7 vaccine.

INTRODUCTION

Gangliosides are sialic acid-containing glycosphingolipids that have increased surface membrane expression on cancers of neuroectodermal origin, including malignant melanoma. There have been a number of immunotherapy studies targeted to gangliosides (1, 2). One of these trials reported improved survival in a small Phase III trial for stage III melanoma patients randomized to adjuvant therapy with the ganglioside GM2 (3). One limitation of vaccination with gangliosides has been the requirement to covalently link the ganglioside to keyhole limpet hemocyanin mixed with a potent adjuvant to produce more potent IgM and IgG responses (3–5). Another limitation of gangliosides is their expensive and difficult purification process. An alternate approach to ganglioside vaccination is the generation of anti-idiotype antibodies that mimic the ganglioside. One such anti-idiotype antibody, which is currently in clinical trials and is designated BEC-2, mimics disialoganglioside GD3 (6, 7). We have been interested in the anti-idiotype approach to a number of antigens (8–12) and have generated an anti-idiotype antibody, designated 1A7, that mimics disialoganglioside GD2 (13, 14). GD2, similar to GM2, is expressed on melanoma and other neuroectodermal tumors, with only minimal expression on normal tissues. Here, we describe the first 12 patients vaccinated with 1A7 mixed with the QS-21 adjuvant.

PATIENTS, MATERIALS, AND METHODS

Patients and Treatment Schedule. The 12 patients presented were the first to be entered into this trial and had American Joint Committee on Cancer stage IV melanoma with measurable metastatic disease (Table 1). All patients were treated with 1, 2, 4, or 8 mg of 1A7, mixed with 100 μg of QS-21. Those patients treated with 8 mg were given two separate injections at each vaccination due to the larger volume. All patients have been on trial from 2 to 23 months and have had...
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forms approved by the University of Kentucky Institutional Review Board. All patients signed informed consent.

sufficient time to generate immunological responses. The vaccine was injected s.c. every other week, four times, followed by monthly injections. Patients were evaluated for disease progression at the end of the fourth injection and every three months thereafter. At the time of disease progression, patients were removed from the study. All patients signed informed consent forms approved by the University of Kentucky Institutional Review Board.

Cell Lines. The mouse thymoma cell line EL-4 and the human colorectal cancer-derived cell line LS174-T were grown in DMEM supplemented with 10% FCS, 2 mm L-glutamine, 100 units/ml penicillin (Life Technologies, Inc.), and 100 μg/ml streptomycin (Life Technologies, Inc.). EL-4 cells that express GD2 on their cell surface membrane were used as positive controls, and LS174-T cells, which lack this antigen, served as negative controls.

Generation of Anti-Idiotypic Antibody for the Clinical Trial. The murine monoclonal IgG2a antganglioside GD2 antibody 14.G2a (Ab1) was used to immunize syngeneic BALB/c mice (15). Hybridoma fusion, cloning, and selection of the monoclonal anti-idiotypic 1A7 (Ab2), as well as production of ascites in bulk quantities in mice, were performed as described previously (10, 11). lA7 was purified from ascites by SDS-PAGE, high-pressure liquid chromatography, and isoelectric focusing.

Adjuvant. QS-21 (kindly provided by Aquila Biopharmaceuticals, Inc., Worcester, MA) is a saponin extract from the bark of the South American soap bark tree Quillaja saponaria monilia and represents an adjuvant approved by the United States Food and Drug Administration for use in humans in experimental vaccine therapy.

Assay for Humoral Immunity. Sera from immunized patients were tested for the presence of anti-anti-idiotypic antibodies. Sera were preincubated with normal mouse immunoglobulin to block human antibodies against isotypic and allotypic determinants and then checked for the presence of anti-anti-idiotypic (Ab3) by reaction with the immunizing antidiontype (1A7) coated onto microtiter plates. Unrelated Ab2 served as control. After washing, the antigen-antibody reaction was tagged using a 125I-labeled anti-idiotypic reagent in a homogeneous sandwich RIA. Pretreatment sera were also used as controls in these assays.

Inhibition of Ab2 Binding to Ab1. Preimmune and hyperimmune patients' sera samples were treated with normal mouse immunoglobulin to remove anti-isotypic and allotypic reactivities. Serial dilutions of sera were then tested for inhibition in the Ab1-Ab2 binding assay. All assays were performed in triplicate. For direct binding inhibition between Ab1 and Ab2, purified Ab1 14.G2a was used to coat plates (500 ng/well), and the binding of radiolabeled 1A7 (Ab2) to Ab1 was tested for inhibition in the presence of different patients' hyperimmune Ab3 sera. Results from this assay indicated whether the Ab3 in patients' sera shared idiotypes with 14.G2a (Ab1). In addition, this inhibition assay demonstrated whether the Ab3 was a true anti-idiotypic.

Purification of Anti-Anti-Idiotypic Antibodies (Ab3) from Hyperimmunized Patients' Sera. Ab3 was purified from the sera of immunized patients by an immunoadsorbent column consisting of immunizing anti-idiotypic immunoglobulin (1A7-IgG1) coupled to Sepharose 4B. Protein bound to this column was eluted with glycine- HCl (pH 2.7), neutralized to pH 7.0 with 3 m Tris, and dialyzed against PBS. This material was then passed over a mouse immunoglobulin immunoadsorbent column of Sepharose 4B to remove anti-isotypic and anti-allotypic reactivities. Antibody that passed through this column was concentrated and used as purified Ab3.

Determination of Immunoglobulin Isotypes and Subclasses. Microtiter plates were coated with GD2 and incubated with patients' purified Ab3 at different dilutions. The isotype or subclass of the bound antibody was determined by ELISA using antihuman isotype and subclass specific reagents (Southern Biotech, Birmingham, AL).

<table>
<thead>
<tr>
<th>Patient no</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Prior therapy</th>
<th>Sites of disease</th>
<th>No. of treatments</th>
<th>Dose (mg)</th>
<th>No. of days on study without progress</th>
<th>No. of days on study to progression</th>
<th>No. of days on study to death</th>
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<td>M</td>
<td>Surgery</td>
<td>Massive abdominal adenopathy</td>
<td>1</td>
<td>1</td>
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<td>M</td>
<td>Surgery</td>
<td>Extensive bone (spine, ribs)</td>
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<td>1</td>
<td>684+</td>
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<td>N/A</td>
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<td>57</td>
<td>M</td>
<td>Surgery, IFN-α</td>
<td>Lymph nodes</td>
<td>4</td>
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<td>627+</td>
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<td>N/A</td>
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<td>Surgery</td>
<td>Parotid gland, lymph nodes, pericardium, spleen</td>
<td>4</td>
<td>8</td>
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<td>Surgery</td>
<td>Nose and sinuses</td>
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<td>14</td>
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<td>7</td>
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<td>47</td>
<td>F</td>
<td>Surgery, IFN-α</td>
<td>s.c.</td>
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<td>14</td>
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<td>9</td>
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<td>Surgery</td>
<td>Lung, bowel, lymph nodes</td>
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<td>4</td>
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<td>M</td>
<td>Surgery, IFN-α</td>
<td>s.c., extensive lymph nodes</td>
<td>2</td>
<td>12</td>
<td>390+</td>
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<td>N/A</td>
</tr>
</tbody>
</table>

* N/A, not applicable.

a Refused further therapy.
Inhibition of Binding of Ab1 to a GD2-positive Cell Line by Ab3. To determine whether crude Ab3 sera or purified Ab3 antibodies are able to compete with Ab1 for binding to the GD2-positive mouse thymoma cell line EL-4, binding of radiolabeled mAb 14.G2a to EL-4 cells was tested for inhibition in the presence of various dilutions of patients’ immune sera. A, patients 1–7; B, patients 8–12.

**Fig. 1** Inhibition of Ab2 binding to Ab1 by patients’ immune sera. Purified Ab1 14.G2a antibody was used to coat the plate (500 ng/well), and the binding of radiolabeled Ab2 1A7 (~90,000 cpm) to 14.G2a was tested for inhibition in the presence of various dilutions of patients’ immune sera. A, patients 1–7; B, patients 8–12.

**Inhibition of Binding of Ab1 to a GD2-positive Cell Line by Ab3.** To determine whether crude Ab3 sera or purified Ab3 antibodies are able to compete with Ab1 for binding to the GD2-positive mouse thymoma cell line EL-4, binding of radiolabeled mAb 14.G2a to EL-4 cells was tested for inhibition in the presence of different dilutions of purified Ab3 or Ab1. Percentage inhibition measured in these assays was calculated according to the formula described previously (16).

**GD2 Binding Inhibition Assay.** Purified GD2 antigen (500 ng/well) was coated onto a 96-well plate. After blocking with 1% BSA in PBS, different dilutions of purified Ab3 or Ab1, along with radiolabeled 14.G2a antibody (~90,000 cpm), were added, and the mixtures were incubated 4 h at room temperature. After washing, bound radioactivity was measured.

**Binding of Ab3 to Purified GD2.** Anti-GD2 reactivity was determined in the purified Ab3 with different gangliosides (GM1, GM2, GM3, GD3, GD2, and GT1b) that were each 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 room temperature with shaking. After washing, the bound antibodies were detected, with alkaline phosphatase-conjugated goat antihuman IgG and goat antimouse IgG serving as second antibodies. The isotype of the antibodies was determined with anti-isotype-specific reagents (Southern Biotech) such as goat antihuman IgG (‘y-chain specific) and goat antihuman IgM (‘m-chain specific).

**Flow Cytometric Analysis with Ab1 and Ab3.** GD2-positive thymoma EL-4 cells (5 x 10⁵ cells per tube) and GD2-negative colorectal cancer LS174-T cells (5 x 10⁵ cells per tube) were reacted with Ab1 (14.G2a) or purified Ab3 at 4°C for 2 h. After washing, the cells were incubated with either goat antihuman F(ab’2)-lgG-FITC (‘y-chain specific) or goat-antihuman F(ab’2)-IgM-FITC (‘m-chain specific) labeled antibody (Tago Immunochemicals, Burlingame, CA) 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).

**Immunoperoxidase Staining of Tumor Sections with Ab1 and Ab3.** The reactivities of Ab1 and purified Ab3 at 50 μg/ml solution were compared on surgical specimens of melanomas by a very sensitive staining method (biotin-streptavidin reagents; Vector, Burlingame, CA) as described in detail elsewhere (17). All sections were counterstained with Meyer’s hematoxylin. Pertinent specificity tests were performed, including blocking of endogenous peroxidase, omission of the first layer, and substitution of nonimmune homologous serum for the specific antiserum. P3–653 melanoma culture supernatant was used as a control.

**ADCC Assay.** GD2-positive M21/P6 cells were labeled in 1 ml of complete DMEM containing 200 μCi of ⁵¹Cr 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 x 10⁵ in 25 μl) were added to individual wells of a 96-well microtiter plate, together with different dilutions of crude immune serum (neat and 1:5). 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

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3 The abbreviations used are: ADCC, antibody-dependent cellular cytotoxicity; MPG, melanoma-associated chondroitin sulfate proteoglycan.
of Ab1 binding to purified disialoganglioside GD2 by purified Ab3 was also found in seven of seven patients and was comparable in some patients (patients 1 and 4) to that achieved with equal quantities of purified Ab1 (Fig. 3). To test whether the patients' Ab3 antibodies represented a true Ab1 anti-GD2 response, they were tested against purified GD2 and GD3 gangliosides, indicating specific binding to GD2 (Fig. 4). The isotype specificity of the Ab3 antibody was predominantly IgG, with minimal IgM (Fig. 5). All subclasses of IgG1 were represented. IgG1 was generally the predominant subclass, with minimal IgM (Fig. 5). All subclasses of IgG1 were represented.

RESULTS

Immune Responses to Anti-Idiotype Vaccine. The development of humoral immunity induced by immunization with Ab2 1A7 mixed with the QS-21 adjuvant was assessed by testing the sera obtained from patients before therapy and following at least four vaccinations. Hyperimmune sera from all 12 patients studied demonstrated an anti-anti-idiotypic-Ab3 response, as indicated by the ability of patients' immune sera to inhibit Ab2 binding to Ab1 (Fig. 1). The immune response appeared independent of whether the patients received 1, 2, 4, or 8 mg of 1A7. In Fig. 1, crude sera from all of the patients, beginning at a dilution of 1:10, showed inhibition. The majority of patients demonstrated nearly 100% inhibition through five serial dilutions. Patients 5 and 11 had lower inhibition but also had fewer immunizations. To further characterize this anti-idiotype response, patients' Ab3 antibodies were purified over immunoadsorbent columns consisting of the immunizing anti-idiotype immunoglobulin adsorbed to Sepharose 4B. Purified Ab3 from seven of seven patients studied inhibited binding of Ab1 to a GD2-positive cell line (EL-4) starting at a concentration of 2.5 μg through 8-fold serial dilutions (Fig. 2). Inhibition of Ab1 binding to purified disialoganglioside GD2 by purified Ab3 was also found in seven of seven patients and was comparable in some patients (patients 1 and 4) to that achieved with equal quantities of purified Ab1 (Fig. 3). To test whether the patients' Ab3 antibodies represented a true Ab1 anti-GD2 response, they were tested against purified GD2 and GD3 gangliosides, indicating specific binding to GD2 (Fig. 4). The isotype specificity of the Ab3 antibody was predominantly IgG, with minimal IgM (Fig. 5). All subclasses of IgG1 were represented.

IgG1 was generally the predominant subclass, with minimal IgM (Fig. 5). All subclasses of IgG1 were represented.
Table 2 Immunoglobulin subclasses

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>IgG1</th>
<th>IgG2</th>
<th>IgG3</th>
<th>IgG4</th>
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<tr>
<td>1</td>
<td>0.98</td>
<td>0.76</td>
<td>0.42</td>
<td>0.49</td>
</tr>
<tr>
<td>2</td>
<td>0.99</td>
<td>0.82</td>
<td>0.61</td>
<td>0.33</td>
</tr>
<tr>
<td>3</td>
<td>0.62</td>
<td>0.63</td>
<td>0.48</td>
<td>0.29</td>
</tr>
<tr>
<td>4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>1.1</td>
<td>0.87</td>
<td>0.91</td>
<td>0.62</td>
</tr>
<tr>
<td>6</td>
<td>1.3</td>
<td>0.72</td>
<td>0.41</td>
<td>0.32</td>
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<tr>
<td>7</td>
<td>0.82</td>
<td>0.91</td>
<td>0.63</td>
<td>0.44</td>
</tr>
<tr>
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<td>1.5</td>
<td>0.63</td>
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<tr>
<td>9</td>
<td>2.45</td>
<td>0.43</td>
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<td>0.85</td>
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<tr>
<td>10</td>
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<td>0.52</td>
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<tr>
<td>12</td>
<td>1.12</td>
<td>0.69</td>
<td>0.82</td>
<td>0.67</td>
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</table>

* Microtiter plates were coated with GD2 and then incubated with 1 µg of purified Ab3 from 1A7 immunized patients' sera. The reaction was developed by ELISA, as described in "Materials and Methods."

**Fig. 5** Isotype analysis of purified Ab3 by ELISA. The plates were coated with ganglioside GD2 and incubated with 1 µg of purified Ab3 and then developed with antihuman IgG (γ-chain specific) and antihuman IgM (μ-chain specific) as second antibodies.

**Fig. 6** Immune flow cytometry of GD2-positive EL-4 cells with patients' purified Ab3. A, EL-4 cells were reacted with preimmune patient's IgG and purified patient's Ab3. B, EL-4 cells were reacted with PBS control and Ab1 14.G2a. C, GD2-negative LS174-T cells were reacted with preimmune patient's IgG and purified patient's Ab3. Representative data from one patient are presented.

**DISCUSSION**

There are a variety of active specific vaccine approaches that have been used for the treatment of malignant melanoma. These include allogeneic irradiated cells or lysates (19–21), autologous cells (22–25), viral oncolysates (26–29), gangliosides (3–5), peptides, and gene therapy (30–33). This subject has recently been reviewed (34). We have used an anti-idiotypic antibody vaccine approach, similar to those used by other investigators. Thus, in one such study, 26 patients with metastatic melanoma were treated with an anti-idiotypic antibody that mimics the MPG antigen. Although these authors did not report whether this anti-idiotypic antibody induced anti-MPG re-
Fig. 7 Immunoperoxidase staining of autologous and allogeneic melanoma tumor tissues by Ab1 and patients' purified Ab3. Serial sections were stained with: Ab1 14.G2a (50 μg/ml; A); patient's purified Ab3 (50 μg/ml) on autologous tumor (B); Ab3 purified (50 μg/ml) from patient treated with an unrelated anti-idiotype antibody (11D10) on tumor section, as in A (C); or patient's purified Ab3 (50 μg/ml) on allogeneic tumor (D).

Fig. 8 ADCC of the GD2-positive cell line M21/P6 as target cells mediated by patient Ab3 sera and normal human peripheral blood mononuclear cells as effector cells. 51Cr-labeled M21/P6 cells were incubated with different patients' sera (neat) in the presence of normal human peripheral blood mononuclear cells at an E:T ratio of 100:1 for 4 h at 37°C. Percentage lysis from natural killer cells was deducted from the above value for each patient. - - - - - - - - lysis at 12%; anything over 12% was considered positive.

responses, they reported human antimouse antibody and anti-anti-idiotype responses. These immune responses correlated with clinical responses in a limited number of patients (35). Similar results have been reported by another group using yet another anti-idiotype antibody that mimics MPG (36, 37).

The most extensive experience with gangliosides in vaccination studies has been observed with ganglioside GM2, covalently linked to keyhole limpet hemocyanin plus the QS-21 adjuvant. The investigators using this approach demonstrated IgM and IgG antibodies against GM2 in the majority of immunized patients. They further showed that these antibodies mediated ADCC. These investigators also reported improved survival in a small randomized Phase III trial for stage III melanoma patients (3). On the basis of these data, GM2 is currently being tested in a large national randomized Phase III trial. Investigators using an anti-idiotype antibody (BEC2) that mimics ganglioside GD3 were able to elicit anti-GD3 antibodies in only 1 of 20 advanced melanoma patients treated with BEC2 without adjuvant (6). In a second clinical trial in melanoma patients who were free of disease following surgical resection, 3 of 14 patients immunized with BEC2 mixed with BCG generated anti-GD3 responses, but 0 of 6 patients immunized with BEC2 and QS-21 generated an anti-GD3 response (7). In contrast, all patients in our study generated an IgG against the GD2 ganglioside, as demonstrated by inhibition assays and direct binding to GD2 and GD2-positive tumor cells. IgG1 was the predominant subclass. Five of five patients' Ab3 antibodies mediated ADCC. One patient had complete resolution of soft
tissue disease, and six patients have not had tumor progression during time intervals ranging from 9 to 23 months.

The murine Ab1 antibody 14.G2a generated the Ab2 1A7 anti-idiotypic antibody used in this study. Interestingly, severe abdominal toxicity requiring morphine was reported in patients following the i.v. infusion of 14.G2a, possibly secondary to GD2 expression on peripheral abdominal nerves (18). We were concerned that generation of a continuous high titer human anti-GD2 would lead to a similar toxicity; this was not observed. Similar to our other anti-idiotypic trials, toxicity was limited to transient local cutaneous swelling associated with mild pain and fevers lasting 12–24 h.

Our experience with the 1A7 anti-idiotypic antibody demonstrates that it is very well tolerated and has a high efficiency of generating true Ab1' responses (anti-GD2) that mediate ADCC, which is the primary immunological goal of such a clinical trial. Ongoing trials include studies with stage III melanoma and small cell lung cancer patients. In future trials, we will attempt to enhance the antitumor responses by adding interleukin 2, granulocyte macrophage colony-stimulating factor, and other cytokines in combination with the 1A7 vaccine.

REFERENCES


Antibody responses in melanoma patients immunized with an anti-idiotype antibody mimicking disialoganglioside GD2.

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