Sequential Immunization of Melanoma Patients with GD3 Ganglioside Vaccine and Anti-Idiotypic Monoclonal Antibody That Mimics GD3 Ganglioside

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ABSTRACT
GD3 ganglioside is an attractive target for immunotherapy of melanoma because it is abundantly expressed on all melanomas but not expressed on most normal tissues. Although GD3 has proven to be one of the least immunogenic gangliosides, our recent studies showed that anti-GD3 antibodies can be induced in patients immunized either with GD3-lactone-KLH (GD3-L-KLH) plus QS-21 adjuvant or with BEC2 anti-idiotypic monoclonal antibody vaccine, which mimics GD3, plus Bacillus Calmette-Guérin. We compared the immunogenicity of these two vaccines and tested whether one vaccine could prime an antibody response to the other. This is the first clinical trial immunizing patients with both antigen and anti-idiotypic monoclonal antibody vaccine. Twenty-four melanoma patients were randomized to be immunized with either BEC2 followed by GD3-L-KLH or in the opposite order. Our prior study suggested that a 25-µg dose of BEC2 was more immunogenic than our standard dose of 2.5 mg and therefore was used in this trial. Overall, 10 of 24 patients (42%) developed anti-GD3 antibodies detectable by ELISA, five in each cohort. All antibody responses were in response to the GD3-L-KLH vaccine. We found no evidence of priming by either vaccine. Antibody responses did not correlate with survival outcomes. Cellular responses were detected by enzyme-linked immunospot against BEC2, Bacillus Calmette-Guérin, and KLH, but not against GD3. We confirmed that GD3-L-KLH vaccine induces anti-GD3 antibodies, but we were unable to confirm our previous finding that a 25-µg dose of BEC2 is immunogenic. Future multivalent ganglioside vaccines should include the GD3-L-KLH vaccine.

INTRODUCTION
GD3 ganglioside is an attractive target for immunotherapy in melanoma because it is abundantly expressed on virtually all melanomas but has extremely limited expression on normal tissues. Early trials using tumor cell preparations were unsuccessful in inducing a detectable antibody response against GD3 (1–3). Because of this, we began a series of vaccine trials directed toward immunizing melanoma patients using different GD3 constructs (4, 5). In general, these trials were all negative in that patients did not develop detectable antibodies against GD3, confirming the poor immunogenicity of GD3.

In searching for alternative methods of immunizing patients against GD3, we developed two approaches. In one approach, a mouse anti-idiotypic monoclonal antibody (MAb) that mimics GD3, designated BEC2, was developed (6). Results from several clinical trials indicated that intradermal injection of 2.5 mg of BEC2 mixed with Bacillus Calmette-Guérin (BCG) as an adjuvant could induce anti-GD3 antibody responses in 25% of patients (7–9). Recently, we explored the immunogenicity of different doses of BEC2 (10). Fifty patients were immunized with BEC2 mixed with BCG at one of the following 5 BEC2 doses: 2.5 µg, 25 µg, 250 µg, 2.5 mg, and 10 mg. We found that anti-GD3 antibody responses were induced in 9%, 30%, 22%, 0%, and 0%, respectively, suggesting that lower doses of BEC2, especially 25 µg, might be more immunogenic, although we were puzzled by the lack of immunogenicity of the previously immunogenic dose of 2.5 mg.

Another approach to induce antibodies against GD3 in patients was to manipulate the GD3 molecule itself. Patients were immunized with 30 µg of GD3-lactone conjugated to KLH [GD3-lactone-KLH (GD3-L-KLH)] mixed with adjuvant QS-21 and in all six patients immunized, antibodies against GD3 in titers up to 1:1280 were detected and were shown to bind cell-surface GD3 (11).

We were interested in comparing directly the immunogenicity of GD3-L-KLH vaccine with the immunogenicity of BEC2. Also, we wished to test the hypothesis that the anti-idiotypic MAb BEC2 could prime the host for response to the GD3-L-KLH vaccine. This has been observed in a mouse model against the antigen Escherichia coli polysaccharide (12). In this current trial, melanoma patients who were free of disease but at high risk for recurrence were immunized either with BEC2/BCG followed by GD3-L-KLH/QS-21 or GD3-L-KLH/QS-21 followed by BEC2/BCG. The primary end point was the development of anti-GD3 antibodies. This is the first cancer vaccine
trial in which patients were immunized with both the tumor antigen and an anti-idiotypic MAb vaccine mimicking the antigen. It is also the first cancer vaccine trial comparing these two forms of vaccine directly.

A secondary end point of the trial was to look for cellular responses against GD3. We reported recently that mice immunized against GD3 could mount an NKT cell response specific for GD3 (13). This current trial was our first opportunity to look for evidence of a cellular immune response against GD3 in patients.

**MATERIALS AND METHODS**

**Patient Eligibility**

Patients older than 18 years of age who were free of melanoma after complete resection of American Joint Commission on Cancer stage III or IV melanoma were eligible. Surgery had to be completed between 1 and 12 months before starting the study. No antimelanoma treatment within the previous 4 weeks was allowed, and all patients had to have a normal WBC and platelet count. Patients were excluded who had significant underlying medical conditions, immunodeficiency, or required chronic treatment with steroids, antihistamines, or non-steroidal anti-inflammatory drugs. Patients who had undergone splenectomy or radiation to the spleen were also excluded. Patients could not have received prior treatment with mouse MAb and could not be pregnant or breast-feeding.

The study was approved by the Memorial Hospital Institutional Review Board and all patients signed written informed consent.

**Vaccine Formulation**

BEC2 was supplied by ImClone Systems, Inc. (Somerville, NJ) as a sterile preservative-free 1.0-ml solution at a concentration of 2.5 mg/ml in PBS. BCG was purchased from Pasteur Merieux Connaught (Toronto, Canada) as a lyophilized powder containing 8 MIE/0.1 ml. BCG was stored at 20 °C until used.

GD3 lactone-KLH/QS-21 (GD3-L-KLH) was prepared at Memorial Sloan-Kettering Cancer Center in the laboratory of Dr. Philip Livingston as described previously (11). Briefly, GD3 purified from bovine buttermilk (Matreya, Inc., Pleasant Gap, PA) was converted to GD3 aldehyde by ozonolysis and conjugated to KLH (Sigma, St. Louis, MO) by reductive amination in the presence of cyanoborohydride. Unreacted GD3 was removed using a Centriprep (30,000 Da cutoff) spin filter. The GD3-KLH was then converted to GD3-lactone-KLH (GD3-L-KLH) by treating with acetic acid. GD3-L-KLH was aliquoted to sterile vials, lyophilized, and stored at −20°C until used.

**Study Design**

Patients were randomized to receive either the BEC2/BCG vaccine followed by GD3-L-KLH/QS-21 (arm A) or GD3-L-KLH/QS-21 followed by BEC2/BCG (arm B). In each arm, there were 4 weeks between completing the first vaccine regimen and starting the second vaccine regimen.

BEC2 was injected intradermally at a dose of 25 μg every other week for four vaccinations, followed by a final vaccination 4 weeks later. We selected this dose based on the results of our previous clinical trial (10) suggesting that this was a more immunogenic dose than the 2.5-mg dose that we had been considering as standard. For each vaccination, BEC2 was mixed with BCG. For the first BEC2 vaccination, a BCG dose of 2 × 107 colony-forming units was administered; for each subsequent BEC2 vaccination, the dose of BCG was decreased 3-fold, as described previously (8). An attenuated BCG dosing schedule was used in patients who had received BCG previously, had a history of tuberculosis, or had a positive purified protein derivative (PPD). GD3-L-KLH was administered at a dose of 30 μg of GD3 mixed with QS-21 (100 μg). It was injected weekly (s.c.) for 4 weeks followed by a booster vaccination 9 weeks later.

**Patient Evaluations**

Before treatment, patients who had no history of having tuberculosis, a positive PPD, or having received BCG, underwent skin testing with PPD. Patients also underwent a complete history, physical exam, complete blood count liver function tests (alkaline phosphatase, AST, total bilirubin), serum lactate dehydrogenase, and chest X-ray. In addition, other radiographic and radionuclide studies were done as deemed necessary by the investigators to confirm that the patient was free of melanoma.

While on the study, patients were examined at least every 6 weeks. Complete blood counts and chemistry screens were repeated at least every 3 months. Chest X-rays or chest computed tomographies were also repeated every 3 months.

The primary end point of the study was the development of an anti-GD3 antibody response. While patients were receiving each vaccine regimen, serum was collected pretreatment, 2 weeks after completing the 4th vaccination and 2 weeks after the 5th vaccination.

A secondary end point of the trial was to detect T-cell responses. Peripheral blood mononuclear cell(s) (PBMC) were collected pretreatment, 2 weeks after the completion of the first vaccine regimen, and 2 weeks after the completion of the second vaccine regimen. PBMCs were cryopreserved for future analysis.

**Immunological Analyses**

**Human Antimouse Antibody (HAMA) Responses.**

The presence of IgG against mouse antibody was detected by ELISA as described previously (16). Briefly, 96-well microtiter plates were coated with BEC2 (1 μg/well) and blocked with 5% milk. Serial serum dilutions were added for 1 h. After washing, alkaline phosphatase-conjugated antihuman IgG was added for 1 h. After washing, the plate was developed with p-nitrophenyl phosphate substrate and absorbance (A) at 405 nm was measured. Titers of HAMA were defined as the highest serum dilution that resulted in A405 nm > 0.05.

**Detection of Ab3 Antibodies.**

HAMA-positive serum obtained after the 5th vaccination with BEC2 were extensively absorbed using mouse IgG conjugated to agarose beads (ICN Biomedicals, Inc.). Serial dilutions of the absorbed sera were then tested by ELISA for binding to BEC2 and to MPC11, an isotype-matched mouse MAb (IgG2b) with framework variable
region sequences almost identical to BEC2 but which has distinct complementarity determining regions (17, 18). Titers to BEC2 in the absorbed serum at least 4-fold higher than titers to MPC11 were considered positive for the presence of Ab3 antibodies specific for hypervariable sequences in BEC2.

IgM and IgG responses to GD3 were detected by ELISA as described previously (16). Briefly, serial serum dilutions were added onto 96-well plates coated with GD3 and blocked with 5% chicken serum. Bound human antibodies were detected using alkaline phosphatase-conjugated secondary antibodies specific for human IgM or IgG. An A405 nm cutoff of 0.05 was used to determine titer. A positive response was defined by an increase in titer of at least 4-fold over pretreatment reactivity. IgG responses to BEC2 were also detected by ELISA as described previously (7, 9).

Sera positive by ELISA were analyzed for binding to cell surface GD3 on melanoma cell line SK-MEL-28 by fluorescence-activated cell sorter using methods described previously (19).

**T-Cell Enzyme-Linked Immunospot (ELISPOT) Assay.**

The methods for ELISPOT assays were essentially as described previously (13) except that antihuman cytokine MAbs were used. Briefly, PBMC to be tested were thawed, washed, and resuspended in X-VIVO 15 serum-free media (Cambrex Bio Science Walkersville, Inc., Walkersville, MD) at a density of $0.5 \times 10^6/0.1$ ml in the presence or absence of GD3 (10 μg/ml), BEC2 (10 μg/ml), BCG (10 μg/ml), KLH (10 μg/ml), or phytohemagglutinin (20 μg/ml). Cells were plated (0.5 × 10^6/ well) in 96-well IP-Multiscreen plates (Millipore, Burlington, MA) coated previously either with antihuman IFN-γ antibodies or antihuman interleukin-4 antibodies (Mabtech Inc., Marie- mont, OH) and blocked with X-VIVO 15. Plates were incubated at 37°C either overnight (for IFN-γ detection) or for 48 h (for interleukin-4 detection). Plates were then extensively washed, and biotinylated anti-IFN-γ or anti-interleukin-4 MAb was added for 2 h. Plates were washed and reacted with avidin-peroxidase complex (Vectastain Elite kit; Vector Laboratories) for 1 h at room temperature. After washing, plates were developed with 3-amino-9-ethyl-carbazole substrate in H2O2 at room temperature for 4 min, then the reaction was stopped by washing plates under running water. After the plates were dry, spots were counted by using a stereomicroscope at a 40-fold magnification and an automated ELISPOT reader system (Carl Zeiss Vision, Germany) with KS ELISPOT 4.0 software.

Patients were considered evaluable for response to GD3, BEC2, BCG, or KLH (a) if PBMC collected before and after immunization against each protein showed adequate viability as measured by trypan blue dye exclusion (>80%) and responsiveness to phytohemagglutinin stimulation, and (b) if there was no preimmunization ELISPOT reactivity. PBMC collected before and after either BEC2 or GD3-L-KLH were evaluated for GD3 responses.

**Patient Evaluable**

All patients who received at least one immunization were considered evaluable for toxicity.

Patients on arm A were considered evaluable for an antibody response if they completed all 5 BEC2/BCG vaccinations, received at least 3 doses of GD3-L-KLH/QS-21, and had serum collected within 3 weeks of the last immunization. Patients on arm B were considered evaluable for an antibody response if they received all 5 doses of GD3-L-KLH/QS-21, at least 4 doses of BEC2/BCG, and had serum collected within 3 weeks of the last immunization.

**Biostatistical Analyses**

Survival time, relapse-free survival time, and time-to-develop stage IV were assessed from the time the patient was rendered free of melanoma before starting the study. Survival times were estimated using the Kaplan-Meier method using Prism version 3.03 (GraphPad Software, Inc.).

**RESULTS**

**Patient Characteristics**

A total of 25 patients were accrued to the trial (Table 1); 12 patients were accrued to arm A and 13 patients were accrued to arm B. One of the patients accrued to arm B experienced progression of disease after completing the GD3-L-KLH vaccine but before starting BEC2/BCG. Therefore, another patient was accrued to replace this patient so that there were 12 assessable patients in each arm of the study.

All patients were free of disease after complete surgical resection, which was accomplished between 2 and 11 months (median 5 months) before starting the protocol. Twenty-two of the patients had American Joint Commission on Cancer stage III melanoma. Six of the patients had no involved lymph nodes (N0) but had primary melanomas deeper than 4 mm. Under the staging system in use at the time, they were considered stage III. Three patients had stage IV disease but were free of disease after complete resection of metastases to skin (1 patient), bowel (1 patient), and lung and breast (1 patient).

<table>
<thead>
<tr>
<th>Table 1 Patient characteristics</th>
<th>Number of patients</th>
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<tbody>
<tr>
<td><strong>Gender</strong></td>
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</tr>
<tr>
<td>Men</td>
<td>17</td>
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<tr>
<td>Women</td>
<td>8</td>
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<tr>
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<td>III</td>
<td>22</td>
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<td>IV</td>
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<tr>
<td><strong>Primary tumor location</strong></td>
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<tr>
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<td>9</td>
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<td>2–4</td>
<td>6</td>
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^* At the time this study was carried out, these T4 patients were considered stage III. Under the current American Joint Commission on Cancer classification, these patients would now be considered stage IIB.
Serological Responses

**Induction of Antibody Responses against GD3.** All 24 assessable patients were evaluated for the development of IgM and IgG against GD3. Overall, 10 patients (42%) developed a detectable anti-GD3 antibody response, 5 patients in each arm. Among patients treated on arm A, one patient developed an IgM anti-GD3 response only; four other patients developed an IgG response with no detectable IgM response. Among the patients randomized to arm B, two patients developed only an IgM response, one patient developed both an IgM and IgG response, two patients developed only an IgG response.

In both arms A and B, the anti-GD3 antibody responses were observed in response to the GD3-L-KLH vaccine; we saw no anti-GD3 responses in response to the BEC2/BCG vaccine (Fig. 1). Similarly, there was no evidence that priming with BEC2 led to an enhanced anti-GD3 antibody response to GD3-L-KLH. The peak titers of anti-GD3 antibodies were relatively low; only 5 of 10 responding patients had peak anti-GD3 titers ≥ 1:80.

The positive sera from the patients who developed an anti-GD3 antibody response detectable by ELISA were tested for the ability to bind to cell-surface GD3. In two of the patients, we could detect IgG binding to GD3+ SK-MEL-28 melanoma cells (Fig. 2).

**Induction of HAMA and Ab3 Responses.** After immunization with BEC2/BCG, all 24 patients developed high-titer HAMA with median titers ≥ 1:25,600 (data not shown). We analyzed HAMA-positive sera in each patient for the presence of Ab3 antibodies. We detected Ab3 antibodies in 10 patients (Fig. 3). The presence of Ab3 antibodies did not correlate with the presence of anti-GD3 antibodies, indicating that although Ab3 responses were induced, there were no Ab1’ responses capable of cross-reacting with the original antigen, GD3. Neither did the induction of an Ab3 response predict which patient would develop anti-GD3 antibodies in response to GD3-L-KLH vaccine. In two patients, we tested the Ab3 for binding to GD3 and did not detect anti-GD3 antibodies. This correlated with the results obtained from the unfractionated serum.

**T-Cell Responses**

Table 2 shows the results of the ELISPOT assays. Thirteen of the patients were ineligible by ELISPOT because of poor viability of cryopreserved PBMC. Of the 11 evaluable patients,
none showed a cellular response against GD3. Seven of 11 evaluable patients showed anti-KLH lymphocyte responses whereas only 2 of 12 evaluable patients showed anti-BEC2 lymphocyte responses. Although both patients who had no pretreatment reactivity against BCG developed a T-cell response against BCG after immunization, 12 other patients showed significant pretreatment reactivity against BCG (data not shown). We were interested to note that 2 of these 12 patients had a positive PPD but 10 of 12 of these patients were PPD-negative. Because PPD reactions are considered to represent TH1 responses, this implies that at least the 10 PPD-negative patients, the BCG reactivity we observed in the ELISPOT assay was due to IFN-γ secreting T cells other than TH1 cells, such as CD8⁺ T cells.

Clinical Outcomes

This clinical trial was not designed to address questions regarding clinical outcomes. For all patients, the estimated median relapse-free survival was 22.1 months with 3-year relapse-free survival of 42%. The estimated median time to progress to stage IV melanoma (among the 22 evaluable stage III patients) was 23 months. An estimated 41% of patients were free of stage IV at 3 years. The estimated median overall survival was 45 months with an estimated 3-year survival proportion of 71%.

There were no significant differences observed between the two treatment arms in any of the survival outcomes (data not shown). Similarly, there was no significant difference in survival results between patients who responded serologically by developing an anti-GD3 antibody response and those who did not (data not shown).

Toxicities

Both vaccines were well-tolerated; there were no grade IV toxicities observed. The only grade III toxicity related to vaccine treatment was injection site reaction because of BCG observed in 24 of the 25 patients treated. The one patient who did not have an injection site reaction never received BEC2/BCG because of early progression of disease. There were no autoimmune reactions seen.

DISCUSSION

This is the first cancer vaccine trial in which patients were immunized with both the antigen and an anti-idiotypic MAb mimicking the antigen. The sequential timing of the vaccination scheme also permitted us to compare these two forms of vaccination directly and to test whether one form of vaccination could prime for the other. The results showed that anti-GD3 antibody responses are more frequently induced by GD3-L-KLH/QS-21 vaccine (42% of patients) than by BEC2/BCG (0% of patients). This study confirms our previous observations that GD3-L-KLH/QS-21 vaccine can induce both IgM and IgG responses against GD3. In contrast to our previous observations (11) however, the proportion of patients who responded immunologically in the current trial was significantly lower (42% versus 100%; P = 0.018). The reason for this difference is not clear, but given the fact that the two trials were performed with different batches of vaccine produced 3 years apart with different sources of KLH, it is possible that there was a real decrease in immunogenicity. Also, although the ELISAs used in the two studies were similar, they were performed by different laboratories using different reagents. These differences could account for the different proportion of immunological responders observed.

As expected, all patients developed a HAMA response. In 10 of 24 patients, we were able to detect an Ab3 response within the HAMA response, antibodies that bind to epitopes unique to BEC2 (idiotypes). In this study, we detected no anti-GD3 antibody responses to the BEC2/BCG vaccine meaning that the Ab3 response did not include Ab1 antibodies. Thus, we were unable to confirm our previous results suggesting that the BEC2 dose used in this study, <100-fold our standard dose, is the optimal immunogenic dose (10). The reason for the diminished rate immune response to BEC2/BCG seen in this current trial is unclear because the dose and schedule of the BEC2/BCG were identical in the two trials. Because we could not confirm the immunogenicity of the 25 µg dose and the preponderance of the data, aside from the previous trial (10), demonstrate that the 2.5-mg dose is immunogenic, we believe that future studies should use a BEC2 dose of 2.5 mg.

Another aim of the study was to determine whether either vaccine could prime for the other vaccine. In a mouse model using neonatal mice immunologically tolerant to E. coli polysaccharide, priming with anti-idiotypic MAb rendered the mice tolerant to GD3 antigens. The results of the current study suggest that the 2.5-mg dose is immunogenic, and we believe that future studies should use a BEC2 dose of 2.5 mg.
capable of responding to *E. coli* polysaccharide and induced a state of protective immunity. This early report represents an example of an anti-idiotypic MAb priming for a protective immune response against a non-protein antigen (12). Although our current trial is the first attempt to use an anti-idiotypic MAb vaccine to prime for a response against the original antigen in patients, we found no evidence of priming by BEC2. In addition, among the patients in arm B who developed an anti-GD3 antibody response to GD3-L-KLH/QS-21, we observed no booster effect from subsequent immunization with BEC2/BCG.

In previous studies, we observed that immunization with BEC2 was associated with improved survival compared with historical reference groups despite the observation that only a minority of patients developed detectable anti-GD3 antibody responses (8, 20). This led us to speculate that non-antibody immune effector mechanisms might play a role, and our recent observations that mice can generate a NKT cell response against GD3 (13) led us to look at the cellular responses in our patients. The ELISPOT results confirmed that cellular responses against KLH and BCG could be readily induced. We were not surprised to observe that 12 of 19 (63%) patients with interpretable pretreatment lymphocyte assays had pre-existing cellular responses against BCG. The fact that only 2 of these 12 patients had positive PPD reactions suggests that the anti-BCG reactivity we detected in the ELISPOT was not attributable to TH1 cells. We speculate that these were CD8+ T cells because they uniformly secreted IFN-γ but did not produce interleukin-4.

Among the 11 patients who were assessable for cellular responses against GD3, none developed an anti-GD3 response. It is important to note that when the trial was designed, little was known about the nature or kinetics of NKT cell responses. Recently, it has become clear that NKT responses to glycolipid antigens occur quickly and are often extinguished within a week of vaccination (13, 21, 22). In our study, we sampled PBMC 12 weeks after beginning immunizations and 2 weeks after the last immunization. This may very well be too late to detect NKT cell responses against GD3. Another limitation has to do with the requirements for glycolipid binding to CD1. It has become clear from the X-ray crystal structure of CD1 that glycolipids with two fatty acid tails are favored for presentation (23–25). Although the GD3 might be cleaved from the GD3-L-KLH conjugate by an antigen-presenting cell, the released GD3 would have only a single fatty acid ceramide tail as a result of the KLH conjugation step and would be unlikely to fit efficiently into the CD1 binding site. This initial attempt to detect anti-GD3 NKT cells in patients must be considered inconclusive. In future studies in which NKT cell responses are to be sought, patients should be immunized with intact ganglioside, and PBMC will need to be sampled days after the first or second immunization.

GD3 remains an attractive target for active immunotherapy in melanoma. Future vaccine studies should take advantage of the immunogenicity of the GD3-L-KLH/QS-21 vaccine construct. Given the previously demonstrated immunogenicity of GM2-KLH and GD2-L-KLH in patients (19), a defined, multiganglioside vaccine can now be considered. In future trials, NKT cells against ganglioside antigens should be looked for earlier in the vaccination course.

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REFERENCES

That Mimics GD3 Ganglioside

Antibody

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