Vaccination with a Bivalent G_{M2} and G_{D2} Ganglioside Conjugate Vaccine: A Trial Comparing Doses of G_{D2}-Keyhole Limpet Hemocyanin[1]

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ABSTRACT

Immunization with GMK vaccine (G_{M2} ganglioside conjugated to keyhole limpet hemocyanin mixed with QS-21 adjuvant) induces anti-G_{M2} antibodies in close to 100% of patients. We found previously that anti-G_{D2} antibodies could be induced in some patients using G_{D2}-keyhole limpet hemocyanin + QS-21 (GDK). In this trial, we wished: (*a*) to determine whether immunization with both GMK and GDK vaccines could induce antibodies against both G_{M2} and G_{D2}; and (*b*) to determine the optimal dose of GDK. Thirty-one patients with melanoma or sarcoma who had no evidence of disease after complete surgical resection were immunized with both GMK (30 μg of G_{M2}) and GDK on weeks 1, 2, 3, 4, 12, 24, and 36. Patients were assigned to one of five GDK dose levels (3, 10, 30, 70, or 130 μg of G_{D2}). Anti-G_{M2} IgM or IgG were induced in 97% of patients. The dose of GDK did not affect the anti-G_{M2} response, although at the highest GDK dose level, 3 of 7 patients did not make anti-G_{M2} IgG. GDK was less immunogenic; overall 45% of patients developed either IgM or IgG against G_{D2}. At GDK doses of 30 or 70 μg, 8 of 11 patients (73%) made either IgM or IgG anti-G_{D2} antibodies. We conclude that both GMK and GDK vaccines can induce antibodies against G_{M2} and G_{D2} in a majority of patients and are safe. The optimal dose of GDK appears to be either 30 or 70 μg when administered with GMK vaccine.

INTRODUCTION

Gangliosides expressed on melanoma are attractive targets for immunotherapy. They are abundantly expressed, are not down-regulated when bound by antibody, do not require HLA molecule coexpression for the immune system to react to them, and may play an important role in melanoma-cell adhesion. In addition, it is clear that patients with melanoma can generate antibodies against ganglioside antigens (1–3). Among the gangliosides expressed by human melanomas, G_{M2} appears to be the most immunogenic. Immunization with G_{M2} and BCG adjuvant induced anti-G_{M2} IgM in more than 85% of patients, and the presence of either vaccine-induced or natural antibodies against G_{M2} correlated with an improved relapse-free survival (4). Subsequent studies showed that immunization with G_{M2} conjugated to KLH[4] and mixed with QS-21 (5, 6) adjuvant (GMK vaccine) induced anti-G_{M2} IgM and IgG in close to 100% of patients (3, 7). On the basis of these observations, we determined that GMK vaccine resulted in optimal immunogenicity at a G_{M2} dose of 30 μg.

Because not all melanomas express G_{M2} (8), it seems likely that a maximally effective ganglioside vaccine will need to include multiple gangliosides. G_{D2} ganglioside is expressed in many melanomas and sarcomas, and monoclonal antibodies against G_{D2} can induce antitumor effects in patients with melanoma or neuroblastoma. Although antibodies against G_{D2} can be induced by immunizing patients with G_{D2}-KLH plus QS-21 (9), the optimal G_{D2} dose has not been determined, and it is not known whether a bivalent ganglioside vaccine can induce antibodies against both G_{M2} and G_{D2}.

We carried out a trial using a mixed ganglioside conjugate vaccine consisting of both G_{M2}-KLH and G_{D2}-KLH with the adjuvant QS-21. G_{M2}-KLH was administered at a fixed dose of 30 μg of G_{M2}/immunization with one of five G_{D2}-KLH doses. The primary goals were to assess the anti-G_{M2} antibody responses induced, determine which G_{D2}-KLH doses induced anti-G_{D2} antibodies, and to determine whether this mixed ganglioside vaccine was safe.

MATERIALS AND METHODS

Vaccine Preparation

G_{M2}-KLH and G_{D2}-KLH were manufactured by Progenics Pharmaceuticals, Inc. (Tarrytown, NY) as described previously (3, 10). QS-21 was supplied by Aquila BioPharmaceuticals (Framingham, MA).

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[4] The abbreviations used are: KLH, keyhole limpet hemocyanin; ITLC, immuno-thin layer chromatography.

Patient Eligibility

Patients with American Joint Committee on Cancer stage III or IV melanoma 2 weeks to 1 year after complete surgical resection were eligible for the study. After the first 24 patients had been entered, the protocol was amended to allow patients with metastatic sarcoma. Patients had to be free of disease, older than 18 years of age, and have a Karnofsky performance status ≥80%. Previous radiotherapy, chemotherapy, or immunotherapy were permitted, but patients must have completed treatment >4 weeks before starting the protocol. Patients were required to have WBC ≥3.0 cells/mm^3, platelets ≥100,000/mm^3, total bilirubin ≤2.0 mg/dl, aspartate aminotransferase ≤74 U/l, lactate dehydrogenase ≤400 U/l, and alkaline phosphatase within normal limits. All patients signed written informed consent before participating in the study.

Patients were excluded if they had had another malignancy within the past 5 years (other than basal cell, squamous carcinomas of the skin, or cervical carcinoma *in situ*), or if they had a medical condition which might make it difficult to complete the full course of treatments or to respond immunologically.

Treatment Plan

Before treatment, all patients had a chest X-ray or chest computed tomography showing no evidence of disease. Additional radiographic tests were performed as necessary to confirm that the patient was free of disease. β-human chorionic gonadotropin was measured within 2 weeks of starting vaccination in women of child-bearing potential to make sure they were not pregnant. All patients had an electrocardiogram within 10 months of the start of treatment.

Patients were injected s.c. with a mixture of G_{M2}-KLH, G_{D2}-KLH, and QS-21 (MGV vaccine). Vaccines were administered weekly for 4 weeks, then on weeks 12, 24, and 36. G_{M2}-KLH was administered at a G_{M2} dose of 30 μg; QS-21 was administered at a dose of 100 μg. Initially, patients were randomized to receive one of four G_{D2}-KLH dose levels: 3, 10, 30, or 70 μg of G_{D2}. After the first 24 patients, additional patients were accrued into a fifth G_{D2}-KLH dose level of 130 μg in a nonrandomized manner.

After seven immunizations with G_{M2}-KLH and G_{D2}-KLH, patients were observed until week 60. At that time, if they remained free of disease, they were eligible to receive three additional booster immunizations with G_{M2}-KLH + QS-21 (no G_{D2}-KLH) at 4-month intervals.

Clinical Evaluation

Patients underwent a history and physical exam at Memorial Hospital on weeks 4, 12, 24, and 48, which corresponded to vaccines 4, 5, 6, and 7. In addition, patients were examined at the times of booster immunization (weeks 60, month 18, and month 22). Chest X-rays or chest computed tomograms were obtained on weeks 12, 36, 48, and at months 18 and 22. Toxicity was scored using standard criteria (11).

Serological Evaluations

ELISA. Serum was collected immediately before each vaccination (including pretreatment), as well as 2 and 6 weeks after vaccines four through seven. A final serum was collected on week 60. In patients who received G_{M2}-KLH booster vaccinations in year 2, serum was collected at the time of each vaccination and 1 month later. Anti-G_{M2} and anti-G_{D2} antibodies were measured using an ELISA method in which ganglioside is adsorbed to 96-well polystyrene microtiter plates. Remaining binding sites on the plate were blocked by PBS/Casein/Tween 20 buffer. Serially diluted patient sera or controls were added, and bound antibody was detected using a goat antihuman IgM or IgG antibody (heavy chain-specific) conjugated to alkaline phosphatase. Plates were developed using *p*-nitrophenyl phosphate substrate and absorbance read at 405 nm with a correction of 620 nm. Antibody titer was defined as the highest dilution of patient serum yielding a corrected absorbance of 0.1. Pooled human serum from previously vaccinated patients with a known anti-G_{M2} or anti-G_{D2} antibody titer or pooled normal human serum with no reactivity were used as positive and negative controls, respectively.

Dot Blot Immunoassay. The specificity of the ganglioside antibody response was analyzed by dot blot immune staining. Ganglioside standards G_{M2}, G_{M3}, and G_{D2} (Sigma Chemical Co., St. Louis, MO) were applied to polyvinylidene difluoride membranes using a dot blot apparatus (Bio-Rad Laboratories, Inc., Hercules, CA). Nonspecific binding to the membrane was blocked with PBS/casein/Tween 20 buffer. Immune serum was added and then goat antihuman IgM- or IgG-specific antibody conjugated to horseradish peroxidase. Specific binding was visualized by chemiluminescence staining. Serum was considered positive if the staining was dose-dependent and specific compared with normal control human serum.

Flow Cytometry. Single cell suspensions of G_{M2}^+ melanoma cells (SK-MEL-31) or G_{D2}^+ melanoma cells (SK-MEL-24) were incubated with pre- or postvaccination serum in PBS with sodium azide on ice for 30 min. The cells were washed with complete culture medium and incubated for 30 min on ice with FITC-conjugated rabbit antihuman IgM or IgG. Cells were washed once, resuspended in paraformaldehyde buffer and analyzed on a FACSCaliber (Becton Dickinson). Patients were considered positive if there was a shift in the relative staining intensity of the postvaccination serum of 20% compared with prevaccination serum.

ITLC. G_{M1}, G_{M2}, G_{M3}, G_{D1b}, and G_{D2} ganglioside standards were separated on a 10 × 10-cm silica plate in chloroform:methanol:water (65:35:6) solution and allowed to air dry. Plates were coated in 5% methacrylate/chloroform-hexane (10:90) for 1 min and air-dried in a fume hood for 20 min. Plates were then blocked in PBS/BSA buffer. Pre- and postvaccination sera were added and then peroxidase-conjugated goat antihuman immunoglobulin. Plates were washed and developed with New England Nuclear Enhanced Chemiluminescence Reagent. Patients were considered positive if specific ganglioside staining was seen in the postvaccination serum compared with prevaccination serum.

On the basis of our previous experience (4), a positive serological response against G_{M2} ganglioside was defined as an ELISA titer ≥1:40. For anti-G_{D2} responses, a positive serological response was defined as a titer ≥1:80 as measured by ELISA in the setting of confirmatory positive staining by dot blot, ITLC, or flow cytometry. This more stringent criterion was

Table 1 Patient characteristics

No.	31
Gender	20 men: 11 women
Stage at treatment	
III	24
IV	7
Primary tumor location	
Extremity	10[a]
Trunk	8
Head/neck	5
Vulva	1
Unknown	7

[a] One male patient had a stage IV synovial cell sarcoma arising from the thigh. All other patients on the study had melanoma.

required for anti-G_{D2} responses to be certain that we were not confounded by background binding.

RESULTS

Patients Treated. Between September 1996 and October 1997, 24 patients were entered into the trial and randomized between one of four G_{D2}-KLH dose levels (3, 10, 30, or 70 μg). After October 1997, an additional seven patients were entered at a G_{D2}-KLH dose level of 130 μg. Overall, 31 patients were treated (Table 1). There were 20 men and 11 women. Twenty-four patients had stage III melanoma; six had stage IV. One patient had synovial cell sarcoma metastatic to lung, which had been completely resected.

All patients received the seven immunizations with bivalent MGV vaccine, except for four patients. These four patients progressed after receiving 4, 5, 5, and 6 vaccinations, respectively. All patients are considered evaluable for immunological response.

Of the 27 patients who completed the first seven immunizations, 22 received either one (5 patients), two (4 patients), or three (13 patients) additional vaccinations with GMK alone at 4-month intervals.

Immunogenicity of G_{M2} Ganglioside. Previous studies demonstrated that immunization with GMK at a G_{M2} dose of 30 μg or 70 μg induces anti-G_{M2} IgM antibodies in 100% of patients and IgG antibodies in 85% of patients (7). Fig. 1 shows the peak anti-G_{M2} titers observed in the patients immunized in the current trial. Before starting immunization, three patients (10%) had detectable anti-G_{M2} antibodies (two patients with anti-G_{M2} IgM at 1:40 and 1:80 and one patient with anti-G_{M2} IgG at 1:40). Although this is a slightly higher incidence of natural anti-G_{M2} antibodies than our previous experience of 5% (4), the difference was not statistically significant. The peak anti-G_{M2} titers ranged from 0–1:2560 for IgM and from 0–1:5120 for IgG.

Across all G_{D2} dose levels, 84% of the patients developed IgM anti-G_{M2} antibodies and 84% developed IgG anti-G_{M2} antibodies; 97% of patients developed either IgM or IgG (Table 2). The median peak anti-G_{M2} titers did not differ among the five G_{D2} dose levels, although we did note a slight decrease in the median peak IgM anti-G_{M2} titers as the dose of G_{D2} increased. At the highest G_{D2} dose level, three of seven patients did not develop an IgG response against G_{M2}, and one patient at

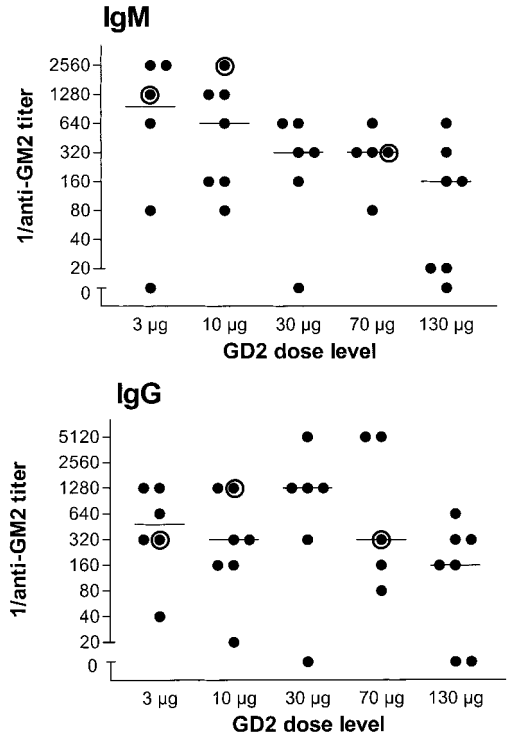

Fig. 1 Peak anti-G_{M2} IgM and IgG antibody titers. Each dot represents peak titers from an individual patient. The *horizontal lines* represent the median titer for each G_{D2} dose level. A titer of ≥1:40 was considered to be a positive anti-G_{M2} response. One patient each in the 10-μg and the 70-μg dose levels had pretreatment IgM anti-G_{M2} titers of 1:80 and 1:40, respectively. One patient in the 3-μg dose level had pretreatment IgG anti-G_{M2} titers of 1:40. These patients are indicated by the *encircled dots*.

the highest G_{D2} dose level did not make either IgM or IgG anti-G_{M2} antibodies. A median of three vaccinations were required to induce peak anti-G_{M2} IgM titers and a median of six vaccinations were required to induce maximum anti-G_{M2} IgG titers. The number of vaccinations required to induce peak anti-G_{M2} titers was not significantly affected by the G_{D2} dose level.

This confirms our previous observations that the GMK vaccine at the G_{M2} dose of 30 μg is highly immunogenic. The addition of the G_{D2}-KLH conjugate vaccine (GDK) did not affect the level of anti-G_{M2} antibody induced nor the number of vaccinations required to induce peak titers, although there was a suggestion that the anti-G_{M2}-antibody response among patients receiving the highest dose of G_{D2}-KLH (130 μg) was less robust in that peak IgM anti-G_{M2} titers were slightly lower and only four of seven patients developed IgG anti-G_{M2} responses.

Immunogenicity of G_{D2} Ganglioside. As expected from our previous experience, G_{D2} was less immunogenic than G_{M2}. Six of 31 patients (19%) developed an IgM anti-G_{D2} response; 12 patients (39%) developed an IgG anti-G_{D2} response. Overall, 14 patients (45%) developed either an IgM or an IgG anti-G_{D2} response (Table 3). No patient had detectable anti-G_{D2} antibodies before immunization. All patients who developed anti-G_{D2} antibodies also developed anti-G_{M2} antibodies. In three patients,

Table 2 Number of patients responding immunologically[a] to G_{M2} at each dose level

G_{D2} dose level (μg)	IgM	IgG	Either IgM or IgG
3	5/6	6/6	6/6
10	7/7	6/7	7/7
30	5/6	5/6	6/6
70	5/5	5/5	5/5
130	4/7	4/7	6/7

[a] Immunological response is defined as a peak antibody titer ≥1:40.

Table 3 Number of patients responding immunologically[a] to G_{D2} at each dose level

G_{D2} dose level (μg)	IgM	IgG	Either IgM or IgG
3	0/6	1/6	1/6
10	1/7	1/7	2/7
30	1/6	4/6	4/6
70	3/5	3/5	4/5
130	1/7	3/7	3/7

[a] Immunological response is defined as a peak antibody titer ≥1:80 confirmed by either dot blot, ITLC, or flow cytometry.

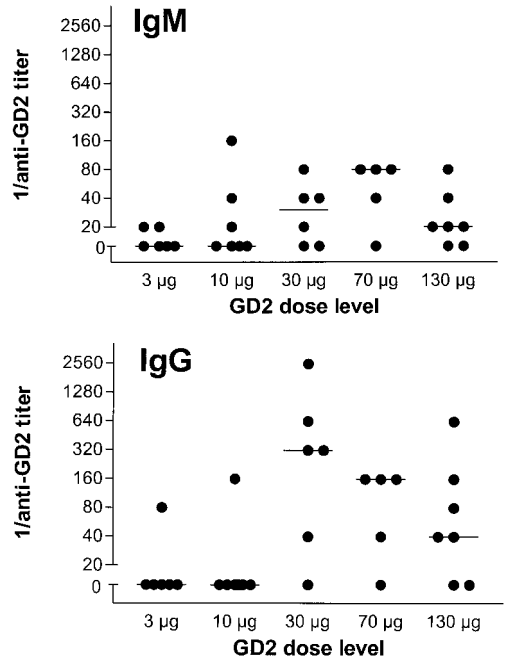

Fig. 2 Peak anti-G_{D2} IgM and IgG antibody titers. Each *dot* represents peak titers from an individual patient. The *horizontal lines* represent the median titer for each G_{D2} dose level. A titer ≥1:80 with confirmatory binding demonstrated by either flow cytometry, ITLC, or dot blot was considered to represent an anti-G_{D2} response.

binding to cell-surface G_{D2} was confirmed by flow cytometry; 12 of 14 patients had binding confirmed by ITLC, which also demonstrated lack of antibody induction against gangliosides G_{M1} and G_{D1b}. The IgM anti-G_{D2} response seemed best at the 70-μg dose level; three of five patients developed IgM anti-G_{D2} antibodies at titers of 1:80 (Fig. 2). The IgG anti-G_{D2} response seemed equivalent at doses ≥30 μg in terms of the proportion of patients developing IgG anti-G_{D2} antibodies (range, 43–67%), although the median peak titers of IgG anti-G_{D2} seemed to be highest at doses of 30 μg and 70 μg (1:320 and 1:160, respectively). Considering the 30-μg and 70-μg cohorts together, 73% of patients (8 of 11) developed anti-G_{D2} IgM or IgG. The median number of vaccinations required to induce peak IgG anti-G_{D2} titers at these two dose levels was six immunizations. From these observations, it appears that when administered with G_{M2}-KLH at a G_{M2} dose of 30 μg, the optimal dose of G_{D2}-KLH vaccine is 30–70 μg.

Toxicity. Vaccines were well tolerated with no grade III/IV toxicity related to treatment. All patients experienced grade II local toxicity at the vaccine sites. Other grade II toxicities were seen in five patients with fatigue/flu-like symptoms, four with fever, and three with headache. There was no neurological toxicity as had been observed in patients infused with monoclonal antibodies against G_{D2} (12–14). We observed no adverse effects associated with a second year of booster immunizations with G_{M2}-KLH.

DISCUSSION

In designing a defined, multivalent ganglioside vaccine, we have built upon our experience immunizing against G_{M2} ganglioside. When patients were immunized with G_{M2} + BCG, 85% developed IgM anti-G_{M2} antibodies but only 10% developed IgG anti-G_{M2} antibodies (4). Still, it was possible to show that, even with this level of immunogenicity, antibodies against G_{M2} (whether natural or vaccine-induced) correlated with an improved relapse-free survival among patients with stage III melanoma who were free of disease after complete surgical resection. This was consistent with previous observations suggesting that naturally occurring antibodies against G_{M2} were associated with improved prognosis (15). Subsequent studies showed that conjugating G_{M2} to KLH and using the adjuvant QS-21 resulted in a more robust, durable, and consistent immune response against G_{M2}. Using this formulation, essentially all patients develop both IgM and IgG antibodies against G_{M2} (7). The hypothesis that higher titers of anti-G_{M2} antibodies and the induction of long-lived IgG anti-G_{M2} antibodies will result in an improved therapeutic effect is currently being addressed in an intercooperative group randomized trial in which patients were randomized to receive G_{M2}-KLH + QS-21 or IFN-α2b after surgery for stage III melanoma.

Because G_{M2} is not expressed on all melanomas, it was logical to build a multivalent ganglioside vaccine that could induce antibodies against even G_{M2}-negative tumors. G_{D2} ganglioside is the next most immunogenic melanoma ganglioside after G_{M2}, and so it was logical to try immunizing patients using both G_{M2}-KLH and G_{D2}-KLH. It was not obvious, however, that the immune response against this combination would be simply additive. In principle, it was possible that one of the gangliosides would prove to be an immunodominant epitope which could prevent an antibody response against the other ganglioside. Because of this, we wished to determine whether it was possible to immunize patients against both G_{M2} and G_{D2}, and to determine what was the optimal dose of G_{D2}.

The results from this study confirmed that a 30-μg dose of

G_{M2} conjugated to KLH is highly immunogenic when given with QS-21; 97% of patients developed antibodies against G_{M2}. For the most part, the immunogenicity of G_{M2} was not affected by the coadministration of G_{D2}-KLH, although at the highest dose of G_{D2}-KLH (130 μg), only four of seven patients developed anti-G_{M2} IgG.

As expected, GDK was less immunogenic than GMK. Overall, only 14 of 31 (45%) patients developed anti-G_{D2} antibodies. It appeared that the 30- and 70-μg dose levels were most efficient in inducing both IgM and IgG anti-G_{D2} antibodies (8 of 11 patients or 73%). At these two dose levels, median peak anti-G_{D2} titers (1:30–1:80 for IgM and 1:160–1:320 for IgG) were lower than the anti-G_{M2} titers. These anti-G_{D2} antibody responses were not associated with the toxicities seen in patients infused with anti-G_{D2} monoclonal antibodies, such as pain or motor weakness (12–14). This was presumably attributable to the much lower antibody levels achieved in our patients immunized with G_{D2}-KLH compared with patients infused with anti-G_{D2} monoclonal antibodies.

We have demonstrated for the first time that it is possible to induce humoral immune responses to two ganglioside targets by simultaneous vaccination in the form of a bivalent ganglioside conjugate vaccine. Although most melanomas express either G_{M2} or G_{D2} (or both), an optimal multivalent ganglioside vaccine would include G_{D3} as well, because essentially all melanomas express G_{D3} and at levels much higher than either G_{M2} or G_{D2}. Attempts to immunize patients against G_{D3} have shown that G_{D3} is far less immunogenic than either G_{M2} or G_{D2}. Despite this, there is evidence that it is possible to induce anti-G_{D3} antibodies in a subset of patients by immunizing either with G_{D3} lactone-KLH (16) or with BEC2, an anti-idiotypic monoclonal antibody that mimics G_{D3} (17). It is hoped that these studies will lead to a multivalent ganglioside vaccine that induces antibodies against the three major melanoma gangliosides: G_{M2}, G_{D2}, and G_{D3}.

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