

# Induction of Antibodies against GM2 Ganglioside by Immunizing Melanoma Patients Using GM2-Keyhole Limpet Hemocyanin + QS21 Vaccine: A Dose-Response Study<sup>1</sup>

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## ABSTRACT

In a previous randomized Phase III trial (P. O. Livingston *et al.*, *J. Clin. Oncol.*, 12: 1036–1044, 1994), we demonstrated that immunization with GM2 and *bacille Calmette-Guérin* reduced the risk of relapse in stage III melanoma patients who were free of disease after surgical resection and who had no preexisting anti-GM2 antibodies. That vaccine formulation induced IgM anti-GM2 antibodies in 74% but induced IgG anti-GM2 antibodies in only 10% of the patients. To optimize the immune response against GM2, a reformulated vaccine was produced conjugating GM2 to keyhole limpet hemocyanin (KLH) and using the adjuvant QS21 (GM2-KLH/QS21). In pilot studies, 70  $\mu\text{g}$  of vaccine induced IgG anti-GM2 antibodies in 76% of the patients. We wished to define the lowest vaccine dose that induced consistent, high-titer IgM and IgG antibodies against GM2. Fifty-two melanoma patients who were free of disease after resection but at high risk for relapse were immunized with GM2-KLH/QS21 vaccine at GM2 doses of 1, 3, 10, 30, or 70  $\mu\text{g}$  on weeks 1, 2, 3, 4, 12, 24, and 36. Serum collected at frequent and defined intervals was tested for anti-GM2 antibodies. Overall, 88% of the patients developed IgM anti-GM2 antibodies; 71% also developed IgG anti-GM2 antibodies. GM2-KLH doses of 3–70  $\mu\text{g}$  seemed to be equivalent in terms of peak titers and induction of anti-GM2 antibodies. At the 30- $\mu\text{g}$  dose level, 50% of the patients developed complement fixing anti-GM2 antibodies detectable at a serum dilution of 1:10. We conclude that the GM2-

KLH/QS21 formulation is more immunogenic than our previous formulation and that 3  $\mu\text{g}$  is the lowest dose that induces consistent, high-titer IgM and IgG antibodies against GM2.

## INTRODUCTION

GM2 is a ganglioside expressed on the surface of most melanomas and has been demonstrated to be immunogenic (1, 2). In our previous studies, we demonstrated that melanoma patients who were free of disease after complete surgical resection and who have natural or vaccine-induced antibodies to GM2 have a decreased risk of relapse (3). Immunization with GM2 alone does not induce antibodies (4); induction of optimal immunity against GM2 requires immunization with a potent adjuvant (5). In previous trials, GM2 was mixed with *bacille Calmette-Guérin*, which resulted in short-lived IgM antibodies (titers  $\geq 1:80$ ) in approximately 74% of patients, but rarely induced IgG antibodies against GM2 (approximately 10% of patients immunized; Ref. 3). Although IgM antibodies are potent mediators of CMC,<sup>4</sup> we hypothesized that the additional induction of an IgG response against GM2 could result in a more pronounced clinical effect. However, induction of IgG antibodies against carbohydrate antigens such as gangliosides would require a T<sub>H</sub> epitope to provide the appropriate signals for immunoglobulin class switching.

To address this issue, GM2 was conjugated to KLH, a carrier protein known to provide T-cell help and administered with adjuvant QS21, a saponin fraction extracted from the bark of the South American tree *Quillaja saponaria Molina* (6). In two pilot studies using GM2 doses of 70  $\mu\text{g}$ , this formulation resulted in high-titer IgG antibodies against GM2 (5, 7). Both IgM and IgG antibodies reacted with GM2<sup>+</sup> tumor cells by flow cytometry and induced complement-mediated lysis (8). In these two trials, 32 (76%) of 42 patients developed IgG antibodies against GM2 at titers  $\geq 1:80$  when doses of QS21  $\geq 100 \mu\text{g}$  were used. Thus, IgG antibodies could consistently be induced against GM2.

The objective of the current trial was to determine the minimal dose of GM2-KLH required for a consistent, high-titer IgM and IgG antibody response. This is one of the first dose-response studies carried out in patients receiving a defined cancer vaccine and identifies a dose that is appropriate for future Phase III trials.

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<sup>4</sup> The abbreviations used are: CMC, complement-mediated cytotoxicity; KLH, keyhole limpet hemocyanin; AUC, area under curve, LDH; lactate dehydrogenase.

Table 1 Dose levels and formulations of GM2-KLH + QS21 vaccine

Dose level ( $\mu\text{g}$ of GM2)	No. of patients immunized
1	5
3	10
10	10
30	20 <sup>a</sup>
70	7
Total	52

<sup>a</sup> The second 10 patients at the 30- $\mu\text{g}$  dose level received vaccine in which the GM2-KLH and QS21 were vialled separately and mixed just prior to administration.

## MATERIALS AND METHODS

### Vaccine Preparation

GM2-KLH was prepared with GM2 from bovine brain and supplied by Progenics Pharmaceuticals, Inc. (Tarrytown, New York) as described previously (5, 9). QS21 was supplied by Aquila BioPharmaceuticals (Framingham, MA).

In general, the vaccine was formulated in a single vial containing both GM2-KLH and QS21. However, a group of 10 patients immunized at the 30- $\mu\text{g}$  dose level were immunized with GM2-KLH and QS21 vialled separately. For these patients, the GM2-KLH and QS21 were mixed by the pharmacist just prior to administration.

### Patient Eligibility

Melanoma patients with American Joint Committee on Cancer stage III or IV, or deep stage II (>4 mm), who were free of disease after complete surgical resection were eligible. All of the pathology was confirmed by the Memorial Hospital Pathology Department. In general, patients were started on vaccine within 10 months of surgical resection, but patients were still eligible even after 10 months if their risk of relapse was felt to be >50%. All of the patients signed written informed consent.

Patients were excluded if their Karnofsky performance status was <80, if they had received systemic therapy or radiotherapy within the previous 8 weeks, or if they had a medical condition that would make it difficult to complete the full course of vaccination or to respond immunologically to the vaccine. Women who were pregnant or breast-feeding were not eligible.

### Treatment Plan

This trial was carried out under an IND from the United States Food and Drug Administration. Within 4 weeks of starting vaccinations, patients had a physical exam, chest X-ray or chest CT, complete blood count, and comprehensive chemistry screen. An electrocardiogram was required within 10 months of starting the study.

Vaccines were administered by the Clinical Immunology nurses (Clinical Immunology Service, Memorial Sloan-Kettering Cancer Center) as a s.c. injection (final volume, 0.75 ml). Vaccinations were administered on weeks 1, 2, 3, 4, 12, 24, and 36.

This study was designed to compare the immunological effects of different doses of GM2-KLH vaccine. Groups of 5–10 patients were accrued to each of five vaccine dose levels in

Table 2 Patient characteristics of 52 patients treated

Stage	
II (>4 mm)	4
III	39
IV	9
Gender	
Male/Female	34/18
Primary site	
Trunk	24
Extremity	20
Head/neck	6
Unknown	2
Median age (range)	60 (26–77)
Median time in months from complete resection until first vaccine (range)	5.7 (2.1–12)

which the GM2-KLH concentration was adjusted to deliver a GM2 dose of 1, 3, 10, 30, or 70  $\mu\text{g}$  (Table 1). All of the vaccinations contained 100  $\mu\text{g}$  of QS21. Subsequently, the vaccine formulation was changed so that the GM2-KLH and QS21 were prepared in separate vials and mixed just prior to vaccine administration. Using this “two-vial system,” an additional 10 patients were immunized at the 30- $\mu\text{g}$  dose level.

### Treatment Evaluation

**Serological Analysis.** Serum was collected immediately prior to each vaccination (including pretreatment), and on weeks 6, 13, 18, 26, 30, 38, and 42. In addition, serum was collected 3 and 6 months after the 7th and final vaccination. Anti-GM2 antibodies were measured using an ELISA method in which GM2 ganglioside is adsorbed to 96-well polystyrene microtiter plates. The 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 phenol substrate, and absorbance was 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-GM2 antibody titer or pooled normal human serum with no anti-GM2 reactivity were used as positive and negative controls, respectively. A positive serological response was defined as an anti-GM2 titer  $\geq$  1:80 observed at two or more time points.

The antibody titers plotted *versus* time were also analyzed as the AUC using Prism version 2.01 software (Graph Pad Software, Inc., San Diego, CA). The AUC of the antibody response was considered to represent the overall exposure to anti-GM2 IgG or IgM over time.

**CMC Assay.** CMC assays were performed by the LDH release method (Boehringer-Mannheim). SK-MEL31 (GM2-positive) or SK-MEL24 (GM2-negative) were plated in 96-well tissue culture plates and incubated at 37°C in a humidified CO<sub>2</sub> incubator. The medium was removed, and plain DMEM containing human serum complement standard (Sigma Chemical Co., St. Louis, MO) was added along with the pre- or postim-

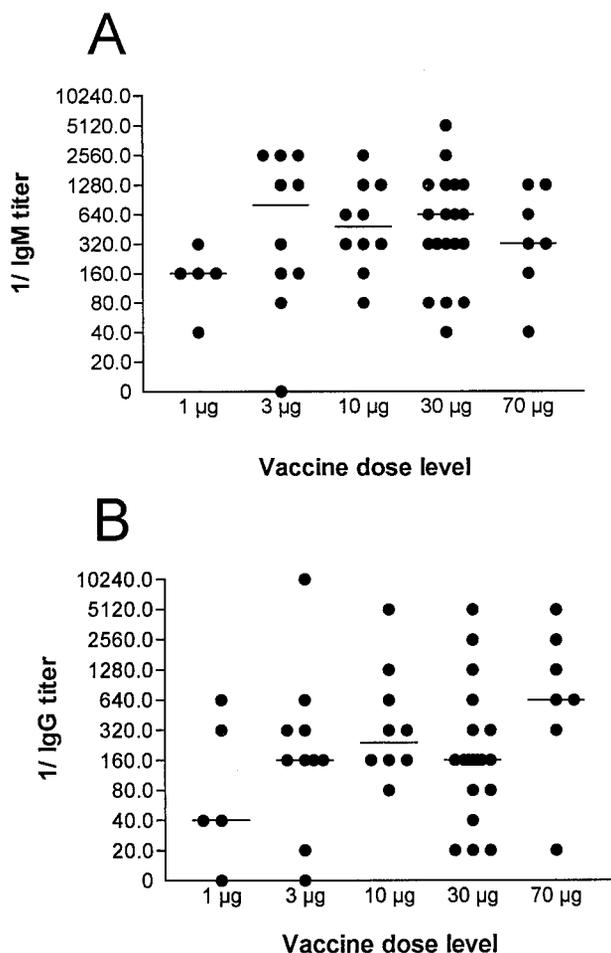


Fig. 1 Peak anti-GM2 antibody titers in patients immunized with GM2-KLH + QS21 at GM2 doses of 1, 3, 10, 30, or 70 µg. Each dot, a single patient. The horizontal lines, the median peak titers for each dose level. A, peak IgM titers; B, peak IgG titers.

munization serum to be tested in duplicate wells. The postimmunization serum tested was the serum sample showing the highest IgM anti-GM2 titers for each patient. Both the complement and serum were used at a final dilution of 1:10. In positive control wells, 1% NP40 was added to measure maximal release. The plate was returned to the incubator for 16 h. The supernatants were removed and transferred to a 96-well ELISA plate for analysis. LDH substrate/catalyst was added, and the plate was incubated in the dark at 25°C for 20 min. The plate was read on a spectrophotometer at 492 nm. Each patient's preimmune CMC reading served as the control for the postimmune CMC result. Percent-specific lysis against each cell line was calculated as follows:

$$\frac{(\text{Postimmune serum LDH release} - \text{preimmune serum LDH release})}{\text{NP40 LDH release}}$$

**Clinical Evaluation.** Patients were evaluated clinically at Memorial Hospital on weeks 12, 24, and 36 and on three months after the 7th vaccination. A chest X-ray, complete blood

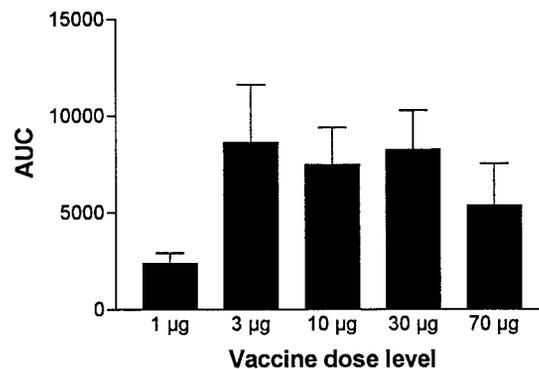


Fig. 2 AUC analysis for the IgM anti-GM2 responses at each dose level. The AUC was calculated for each patient up to week 30. Height of the columns, the mean ( $\pm$  SE) AUC for each dose level.

count, and comprehensive screening profile were repeated at the time of the 5th and 7th vaccination; an electrocardiogram was repeated at the time of the 7th vaccination. Toxicity was scored using standard criteria (10).

## RESULTS

**Patient Characteristics.** Fifty-two patients were entered on this trial between January 1995 and April 1996 (Table 2). There were 34 men and 18 women. Most (75%) of the patients had stage III melanoma; 8% had deep stage II, and 17% had stage IV. The patients had been free of disease for a median of 5.7 months before beginning the trial.

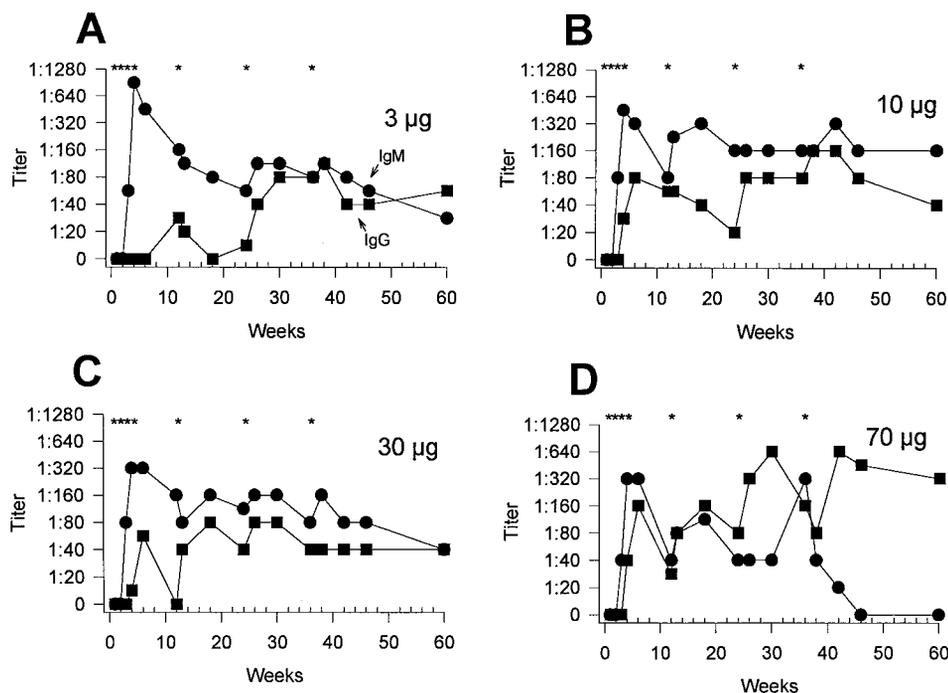
**Serological Results.** Applying rigorous definitions of response (defined in "Materials and Methods") 88% of the patients immunized in this study developed an IgM response against GM2; 71% developed an IgG response. Fig. 1 shows the peak anti-GM2 titers attained at each dose level. For IgM, the median peak titers ranged from 1:160 to 1:800; for IgG the median peak titers ranged from 1:40 to 1:640. When comparing the incidence of nonresponding patients (peak titers  $\leq$  1:40) for IgM and IgG at each of the dose levels, we found no difference for the IgM response ( $P = 0.73$ ;  $\chi^2$ ) or IgG response ( $P = 0.19$ ;  $\chi^2$ ). From the exploratory analysis, it appeared that there were fewer IgG responses at the 1-µg dose level.

An AUC analysis was performed for both IgM and IgG anti-GM2 responses on each patient until week 30, and the mean AUCs at each dose level were compared. For the IgM anti-GM2 response, the mean AUC at the 1-µg dose level was lower than the mean AUC at any of the other dose levels (Fig. 2). The mean AUC for the IgG response was also lower in patients treated at the 1-µg dose level compared with the mean AUCs at the other dose levels (data not shown), but this difference was not statistically significant. There were no differences in the AUC for the other dose levels.

Given that the 1-µg dose level seemed to have a lower incidence of inducing IgG against GM2 and a lower mean AUC for the IgM response, we concluded that the 1-µg dose level was less immunogenic than the other dose levels. As a result, we focused on the 3-, 10-, 30-, and 70-µg dose levels.

Fig. 3 illustrates the median anti-GM2 IgM and IgG titers for patients immunized at the 3-, 10-, 30-, and 70-µg dose

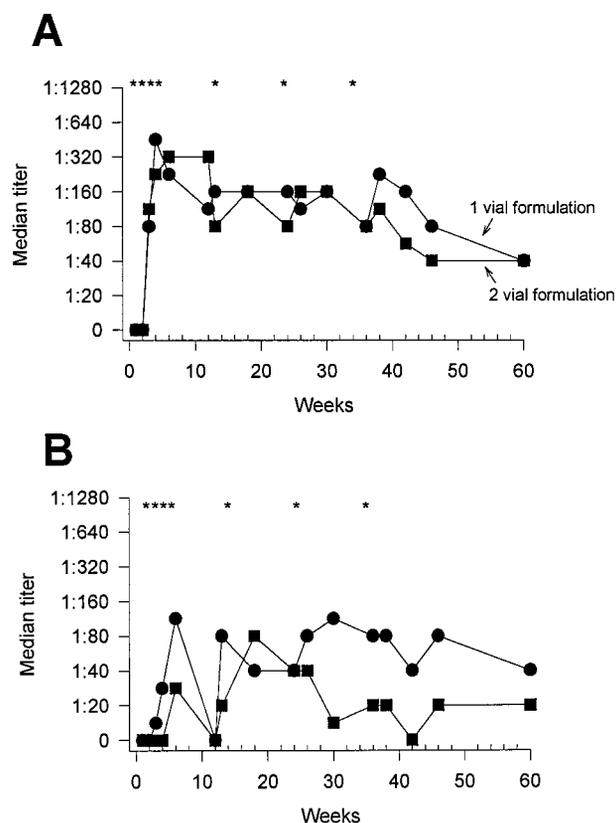
**Fig. 3** Median anti-GM2 antibody titers in patients immunized with GM2-KLH + QS21 at GM2 doses of 3  $\mu$ g (A), 10  $\mu$ g (B), 30  $\mu$ g (C), or 70  $\mu$ g (D). IgM titers (●) and IgG titers (■) are shown separately at each dose level. \*, administration of vaccine.



levels. At these four dose levels, there was a consistent IgM response followed by an IgG response. Both the IgM and IgG responses were sustained for months after the final immunization. At week 60 (5½ months after the last immunization), serum was available on 20 patients who had developed an IgM response and 19 patients who had developed an IgG response. Analysis of these sera showed that the IgM response persisted in 45% of the cases; the IgG response persisted in 53% of the cases (data not shown). This demonstrates that, in one-half of the patients who developed anti-GM2 antibodies, the antibody response persisted for at least 5½ months.

Most of the patients immunized on this trial received vaccine that had been formulated in one vial (*i.e.*, GM2-KLH and QS21 were stored together). However, 10 of the 20 patients immunized at the 30- $\mu$ g dose level received vaccine formulated in two vials because we obtained evidence that the stability of the vaccine was enhanced if the GM2-KLH and QS21 were stored in separate vials and mixed just prior to vaccine administration. We compared the anti-GM2 response induced in patients immunized with the single-vial *versus* the two-vial formulation at the 30- $\mu$ g dose level (Fig. 4). The median IgM titers were similar in the two groups; the median IgG titers were slightly lower in the group receiving vaccine formulated as two vials. All of the patients immunized with the single-vial formulation developed anti-GM2 antibodies, and only one patient immunized with the two-vial formulation failed to develop anti-GM2 antibodies. We conclude that there was no difference in the immunogenicity between the one-vial and the two-vial formulations.

**CMC.** Sera from 18 of the 20 patients treated at the 30- $\mu$ g dose level were available to be tested for the ability to bind melanoma cells and to fix the complement (Fig. 5). In 9 of the 18 patients, the postvaccination sera showed an increase in



**Fig. 4** Comparison of median anti-GM2 IgM titers (A) and IgG titers (B) among patients immunized at the 30- $\mu$ g dose level. ●, patients immunized with vaccine formulated in a single vial; ■, patients immunized with vaccine formulated in 2 vials, in which the GM2-KLH and QS21 vialled separately; \*, administration of vaccine.

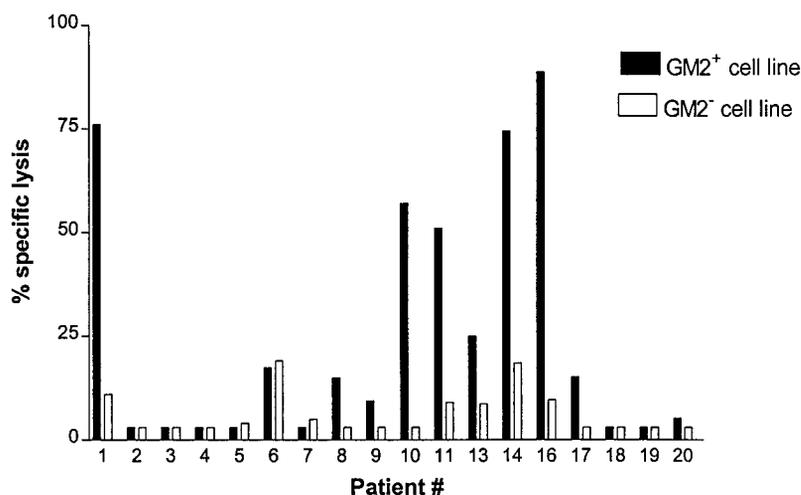


Fig. 5 CMC of sera from patients immunized at the 30- $\mu$ g dose level. ■, the increase of CMC against a GM2<sup>+</sup> cell target in postvaccination sera compared with pretreatment sera. □, the increase of CMC against a GM2<sup>-</sup> cell target in postvaccination sera compared with pretreatment sera. Data for patients 12 and 15 are not available.

CMC compared to pretreatment that was specific for the GM2<sup>+</sup> cell target. In the remaining nine patients, there was either no increase in CMC compared with pretreatment levels (patients 2, 3, 4, 5, 7, 18, 19, and 20) or the increase was not specific for GM2 (patient 6). Induction of complement-fixing activity correlated with a peak IgM anti-GM2 titer of 1:640. All of the nine patients demonstrating CMC activity in their serum had peak IgM anti-GM2 titers  $\geq$  1:640 as opposed to only two of nine patients without CMC activity ( $P = 0.002$ ; Fisher's exact test).

**Toxicity.** Virtually all of the patients experienced inflammation and/or pruritis at the site of injection attributed to the known effects of the QS21 adjuvant (7). Other common side effects were: (a) fever (71%); (b) mild fatigue (44%) and flu-like symptoms (58%); (c) chills (29%); and (d) myalgias (48%). These were self-limiting, never more severe than grade 2, and rarely lasted more than 24 h. Headache was seen in 66% of the patients and was grade 1–2 except in one patient with a grade-3 headache. These toxicities were felt to be due largely to QS21, which is consistent with the observation that there was no correlation between vaccine dose and toxicity. Grade 3 or 4 toxicity possibly related to vaccine occurred in four patients. One patient developed transient dyspnea, which resolved spontaneously. Another patient reported 2–3 days of severe dizziness, which also resolved spontaneously. One patient developed atrial flutter while on the study and required treatment. A fourth patient, with a history of migraine headaches, reported a grade 3 headache associated with vaccine therapy. No patient was taken off study because of toxicity.

## DISCUSSION

The current trial confirms that vaccinating melanoma patients with GM2-KLH + QS21 induces both IgM and IgG antibodies against GM2. We observed that 88% of patients developed IgM anti-GM2 antibodies and 71% developed IgG anti-GM2 antibodies. This compares almost exactly with the immunological results observed in our previous pilot trials (5, 7). Because the previous trials used vaccine produced at Memorial Sloan-Kettering Cancer Center and the current trial used vaccine produced by Progenics Pharmaceuticals, Inc., this dem-

onstrates that subsequent lots of the vaccine can be produced successfully and that the immunogenicity is reproducible. The results also show that the vaccine can be formulated either with QS21 or vialled separately and mixed with QS21 just prior to administration. We favor formulating GM2-KLH and QS21 in separate vials because of improved stability.

This is one of the first cancer vaccine trials to explore dose-response effects using a defined antigen. Our previous trials used GM2-KLH at a GM2 dose of 70  $\mu$ g and demonstrated that all of the patients developed IgM anti-GM2 and 76% developed IgG anti-GM2. In this current trial, we have explored GM2 doses of 1, 3, 10, 30, and 70  $\mu$ g. We conclude that the immunogenicity of GM2-KLH at a GM2 dose of 1  $\mu$ g is suboptimal based on the fact that the 1- $\mu$ g dose was less likely to induce IgG anti-GM2 antibodies. The mean AUC for the anti-GM2 IgM antibody responses was also lowest for the 1- $\mu$ g dose level, which implies that this dose resulted in the lowest level of tumor-cell exposure to anti-GM2 antibody. At the higher vaccine doses (3, 10, 30, or 70  $\mu$ g), there was no apparent difference in the immunogenicity of the vaccine. Peak titers, AUC, antibody responses over 60 weeks, and percent of nonresponding patients were similar at the 3-, 10-, 30-, and 70- $\mu$ g dose levels.

In patients immunized at the 30- $\mu$ g dose level, 50% of the patients developed antibodies that fixed complement and resulted in CMC against GM2<sup>+</sup> melanoma. CMC activity correlated with peak IgM anti-GM2 titers  $\geq$  1:640. This demonstrates that immunization induced anti-GM2 antibodies capable of binding cell-surface GM2 and mediating effector functions.

In at least one-half of the patients, the anti-GM2 antibody response persisted for more than 5½ months. This is consistent with the notion that the KLH carrier protein provides sufficient T-cell help to induce a more prolonged antibody response against GM2. It is also important to note that patients at the 70- $\mu$ g dose level received a 23-fold higher KLH dose compared with patients at the 3- $\mu$ g dose level, and that this was not associated with any excessive toxicity or decreased immunogenicity. This is reassuring as we consider construction of multivalent vaccines containing 4 or 5 antigens conjugated to KLH. Our results suggest that these higher

total KLH doses will neither be more toxic nor lead to diminished immunogenicity.

These studies provide a basis for additional trials with GM2-KLH + QS21. Future clinical trials will examine the effects of IFN- $\alpha$  on the anti-GM2 response induced by GM2-KLH + QS21, the immunogenicity of GM2-KLH + QS21 combined with GD2-KLH, and a Phase III trial comparing GM2-KLH + QS21 to IFN- $\alpha$  for the ability to prevent recurrence of melanoma in stage III patients. For these trials, a vaccine dose  $\geq 3$   $\mu$ g of GM2 should be used.

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