

Consistent Antibody Response against Ganglioside GD2 Induced in Patients with Melanoma by a GD2 Lactone-Keyhole Limpet Hemocyanin Conjugate Vaccine plus Immunological Adjuvant QS-21

Govind Ragupathi,¹ Philip O. Livingston, Chandra Hood, John Gathuru, Susan E. Krown, Paul B. Chapman, Jedd D. Wolchok, Linda J. Williams, Roberta C. Oldfield, and Wen-Jen Hwu

Laboratory of Tumor Vaccinology [G. R., P. O. L., C. H., J. G.] and Clinic Immunology Service [G. R., P. O. L., S. E. K., P. B. C., J. D. W., L. J. W., R. C. O., W-J. H.], Department of Medicine Memorial Sloan-Kettering Cancer Center, New York, New York

ABSTRACT

Purpose: Melanomas, sarcomas, and neuroblastomas abundantly express the ganglioside GD2 on the cell surface where it is susceptible to immune attack by antibodies. Overexpression of GD2 on these tumors is striking, as is the frequency of clinical responses after treatment of neuroblastoma with monoclonal antibodies against GD2. In addition, preclinical models have demonstrated the ability of a GD2-keyhole limpet hemocyanin (KLH) conjugate vaccine to induce antibodies that eliminate micrometastases. However, vaccination of patients with GD2-KLH has previously failed to induce a consistent relevant antibody response. We test here whether the use of GD2 lactone-KLH can overcome the low immunogenicity of GD2-KLH.

Experimental Design: Eighteen patients with melanoma were vaccinated s.c. in the adjuvant setting on weeks 0, 1, 2, 3, 10, and 24. Groups of 6 patients were entered at three dose levels (3, 10, or 30 μ g) of GD2 lactone (GD2L) in vaccines containing GD2L-KLH plus the immunological adjuvant QS-21. Blood was drawn at regular intervals to assess the antibody response.

Results: The vaccine was well tolerated. The majority of patients in all three dose levels produced anti-GD2 antibodies

detectable by ELISA assay. Specificity for GD2 was also confirmed by immune thin-layer chromatography. Although there was no statistical difference in terms of titers between the three groups, patients at the 30- μ g dose level had higher titers and longer lasting antibody responses overall by ELISA (median IgM/IgG peak titer 1:640/1:80) and generated the strongest cell surface reactivity by fluorescence-activated cell sorting (median IgM peak percentage positive cells/mean fluorescence intensity for pre- and post-vaccination sera is 10%/63 and 70%/135). Patients vaccinated with the 30- μ g GD2 dose also had the most potent complement dependent cytotoxicity using human complement, with 5 of 6 patients showing strong cell surface reactivity by fluorescence-activated cell sorting and >30% cytotoxicity by chromium release with a serum dilution of 1/100.

Conclusions: GD2L-KLH conjugate vaccine plus adjuvant QS-21 induces antibodies against GD2 that bind to the cell surface and induce complement-dependent cytotoxicity in the majority of patients with melanoma.

INTRODUCTION

The majority of patients with melanoma and sarcoma who eventually die of their disease can initially be rendered free of detectable disease by local treatments such as surgery, and radiotherapy. Cancer recurrence is a consequence of micrometastasis established before the potentially curative therapy. In a variety of experimental animal models, mAbs² and/or vaccine-induced antibodies against a variety of cell-surface antigens, including gangliosides, can protect against outgrowth of established micrometastasis (1, 2). Murine mAbs against GM2, GD2, and GD3 have also resulted in clinical responses in a proportion of treated patients (3–6). It has been shown that either vaccine-induced or natural antibodies against a variety of antigens, including GM2, correlates with improved disease relapse-free survival (7–10). Melanomas, sarcomas, and neuroblastomas abundantly express the gangliosides GM2, GD2, and GD3 on the cell surface where they are susceptible to immune attack by antibodies (11–13). GD2 is an especially appealing target. Overexpression of GD2, especially on neuroblastomas and sarcomas, is striking and the frequency of clinical responses after treatment

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¹ To whom requests for reprints should be addressed, at Laboratory of Tumor Vaccinology, Department of Medicine, Box 113, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021. Phone: (212) 639-8554; Fax: (212) 794-4352; E-mail: ragupatg@mskcc.org.

² The abbreviations used are: mAb, monoclonal antibody; CDC, complement-dependent cytotoxicity; FACS, fluorescence-activated cell sorting; HSA, human serum albumin; ITLC, immune thin-layer chromatography; GD2L or GD3L, GD2 or GD3 lactone; KLH, keyhole limpet hemocyanin.

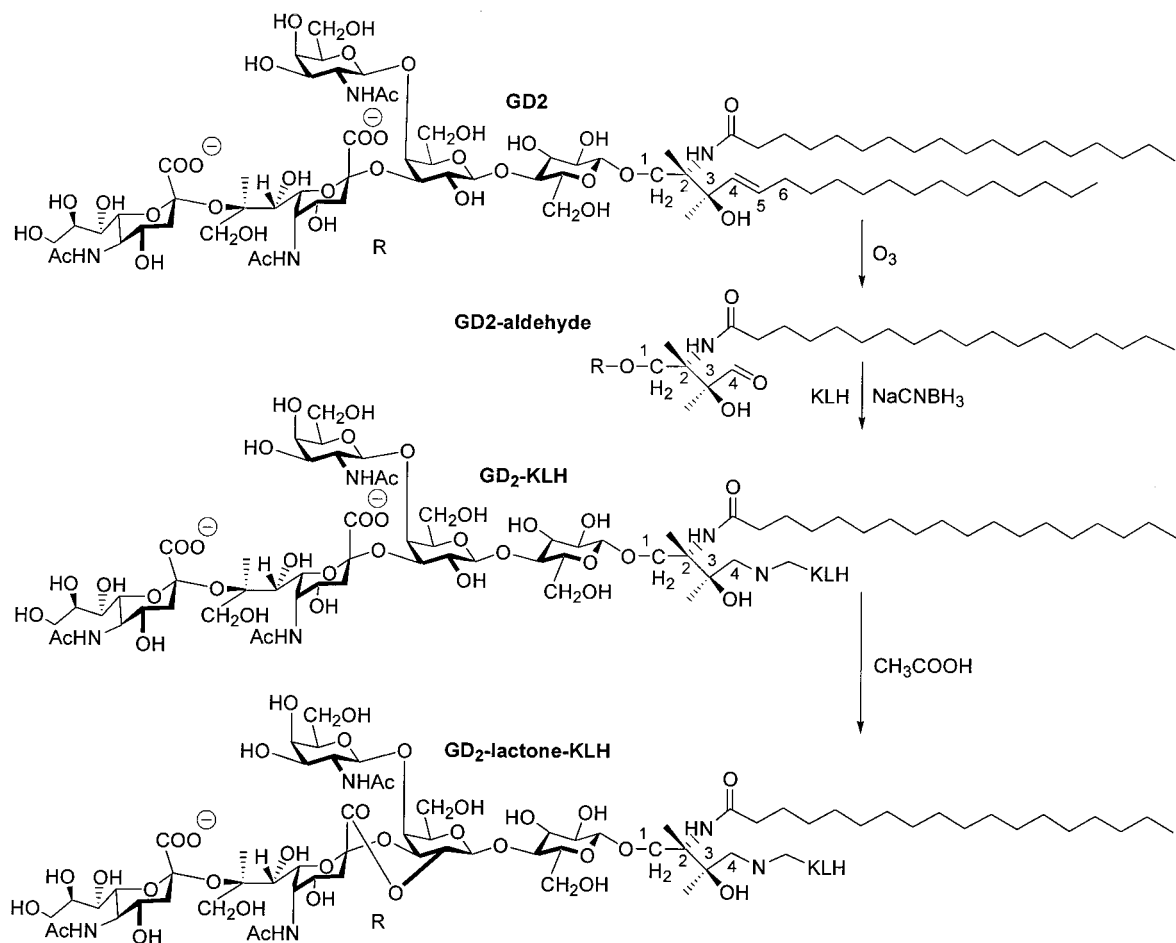


Fig. 1 Structure of GD2 and GD2L-KLH conjugate.

of neuroblastoma patients with mAbs against GD2 is relatively high. In addition, preclinical models have demonstrated that vaccine-induced GD2 antibodies eliminate micrometastases (1).

We have found that the optimal approach for inducing antibodies against gangliosides is to link the ganglioside covalently to KLH and to combine the conjugates with potent saponin immunological adjuvants such as QS-21 (7, 14–16). Vaccination of melanoma patients with GM2-KLH conjugate vaccines containing 10 or 30 μ g GM2 plus QS-21 results in a high titer antibody response in most patients. Although this approach applied to GD2 and GD3 has failed to induce a consistent, relevant antibody response against these gangliosides, antibody responses against GD3 could be increased by conversion of GD3 in the GD3-KLH conjugate to GD3L (17). This approach is based on a previous report by Nores *et al.* (18) demonstrating that GM3 lactone was a more effective immunogen than GM3, presumably as a result of increased rigidity resulting from lactone ring formation (Fig. 1). We have now applied the same approach to immunization against GD2 and report here for the first time the effective induction of antibodies against GD2 and GD2 positive-tumor cell lines in the majority of vaccinated patients.

MATERIALS AND METHODS

Materials. GD2 was prepared by Progenics Pharmaceuticals from bovine brain GD1b by treatment with β -galactosidase as described previously (7). GM1, GM2, GM3, and GD3, sodium cyanoborohydride, 4-chloro-1-naphthol, *p*-nitrophenyl phosphate, and clinical grade KLH were purchased from Sigma Chemical Co. (St. Louis, MO). QS-21 (19) was obtained from Antigenics, Inc. (New York, NY and Framingham, MA). Goat antihuman IgG and IgM conjugated with alkaline phosphatase obtained from Kierkegaard and Perry Labs (Gaithersburg, MD) were used for ELISA. Goat antihuman IgM or IgG labeled with FITC were obtained from Southern Biotechnology Associates Inc. (Birmingham, AL) and used in FACS. Horseradish peroxidase-conjugated goat antihuman IgM and IgG purchased from Tago (Burlingame, CA) were used for dot-blot immune staining and ITLC. Rabbit antimouse immunoglobulins conjugated with horseradish peroxidase were obtained from Zymed (San Francisco, CA) and used for ITLC with mouse monoclonal control antibody 3F8 against GD2 (4). High-performance thin-layer chromatography silica gel plates were obtained from E. Merck (Darmstadt, Germany).

Vaccine Preparation. GD2-KLH conjugate was prepared as described previously for GD3 (Fig. 1; Ref. 17). The principle involved in the conjugation procedure is cleavage of the double bond of ceramide by ozone, generation of an aldehyde group, and conjugation to ϵ -amino groups on lysine of KLH by reductive amination. The GD2-KLH conjugate was prepared in four batches and combined together. Thirty-nine percent of GD2 in the initial reaction mixture was conjugated with KLH. The GD2-KLH molar epitope ratio for the combined preparation was 951/1. Of the vialled GD2-KLH conjugate, >95% of GD2 in the vaccine vial was conjugated to KLH as determined by ITLC.

Because of the unstable nature of GD2L, we prepared the GD2-KLH conjugate first then converted it to GD2L-KLH by acid treatment (Fig. 1) and lyophilized immediately. Briefly, equal volumes of GD2-KLH and glacial acetic acid (v/v) were mixed in a sterile glass tube. To monitor conversion of GD2 to GD2L, the conversion of the 3–4% free GD2 present with the conjugate was determined by TLC. After 4 h at 37°C with gentle shaking when ~80% of the GD2 had been converted to the lactone, the acetic acid was quickly removed using a Centriprep (Amicon; M_r 30,000 molecular cutoff filter) with multiple saline washes. The conjugate was sterilized by passing through a 0.22- μ m filter. The amount of ganglioside in the conjugate was determined by estimating sialic acid content using the resorcinol method (20). GD2L-KLH conjugate was vialled at doses of 3, 10, and 30 μ g of ganglioside and lyophilized under sterile conditions. Before injection, 100 μ g of QS-21 were mixed with the GD2L-KLH as it was reconstituted in normal saline to a total volume of 1 ml.

Patients and Clinical Protocol. Patients with American Joint Committee on Cancer stage II (>4 mm primary) or patients with stage III or IV metastatic malignant melanoma (regional or systematic metastases) who were rendered free of detectable disease and <1 year after surgery were candidates for this trial. No patient had received prior chemotherapy or radiation therapy, and patients requiring treatment with steroidal or nonsteroidal anti-inflammatory drugs were excluded. Six patients were entered at each dose level starting at the lowest and proceeding to the highest. GD2L-KLH conjugate containing 3, 10, or 30 μ g of ganglioside, and 100 μ g of immunological adjuvant QS-21 were mixed immediately before vaccine administration in a total volume of 1 ml of saline. Four vaccinations were administered s.c. at 1-week intervals; two additional vaccinations were administered at weeks 10 and 24. Peripheral blood (20–30 ml) was drawn on weeks 0, 1, 3, 5, 10, 12, 18, 24, 26, 32, and 48 sera were separated and stored at –80°C until tested for the presence of anti-GD2 antibodies. The clinical study was conducted under a protocol approved by the Memorial Sloan-Kettering Cancer Center Institutional Review Board. All patients signed written informal consent. Toxicity was graded according to National Cancer Institute Common Toxicity Criteria.

ELISA. ELISAs were performed as described previously (17). To determine the titers of GD2 antibodies, ELISA plates were coated with GD2 at 0.1 μ g/well in ethanol. Serially diluted patient serum in 1% HSA was added to wells of the coated plate and incubated for 1 h at room temperature. Goat antihuman IgM or IgG conjugated with alkaline phosphatase served as second

antibodies. The antibody titer was defined as the highest serum dilution showing an absorbance ≥ 0.1 over that of normal sera. Immune sera were also tested for nonspecific stickiness on plates that were processed identically but without ganglioside, and the reading was subtracted from the value obtained in the presence of gangliosides.

ITLC. Immune staining of gangliosides with mAb 3F8 (provided by Dr. Nai-Kong Cheung, Memorial Sloan-Kettering Cancer Center) or human sera was performed after separation of purified gangliosides or tumor extracts on high-performance thin-layer chromatography silica gel glass plates as described previously (12, 17). The plates were coated with 1% Plexigum in *n*-hexane, blocked with 1% HSA in PBS for 2 h, and incubated overnight with patient sera diluted with 1% HSA in PBS at various concentrations at room temperature. The plates were washed with PBS containing 0.05% Tween 20 and incubated with antihuman IgG or IgM antibodies conjugated with horseradish peroxidase at 1:200 dilution for 3 h at room temperature. The plates were washed with PBS-0.05% Tween 20 and developed with 4-chloro-1-naphthol with H_2O_2 .

FACS Assay. GD2-positive neuroblastoma cell line NMB-7 served as a target. Single cell suspensions of 2×10^5 cells/tube were washed with 3% FCS in PBS and incubated with 20 μ l of antisera or mAb 3F8 for 30 min on ice. After washing the cells twice with 3% FCS in PBS, 20 μ l of 1:25 goat antihuman IgG or IgM-labeled with FITC were added. The suspension was mixed, incubated for 30 min, and washed. The percent positive population and mean fluorescence intensity of stained cells were analyzed using a FACS Scan (Becton Dickinson, San Jose, CA; Ref. 17). Pre- and postvaccination sera were analyzed together. Prevacination sera were used to set the FACScan result at 10% as background for comparison to percent positive cells with postvaccination sera.

CDC. CDC was assayed at a serum dilution of 1:100 with NMB-7 cells and human complement by a chromium release assay as described previously (14, 17). All assays were carried out in triplicate. Cells incubated only with culture medium, complement, antisera, or mAb 3F8 served as controls. Spontaneous release was calculated based on the chromium released by target cells incubated with complement alone. Maximum release was determined by incubating target cells with complement and 1% Triton X-100. Percent cytolysis was calculated according to the formula: specific release (%) = (experimental release – spontaneous release)/(maximum release – spontaneous release) \times 100.

RESULTS

Clinical Considerations. From January 2001 to August 2001, 18 patients were entered sequentially, 6 patients/group, to receive GD2L-KLH vaccines containing 3, 10, or 30 μ g of GD2L plus 100 μ g of QS-21. All had stage II to III or IV metastatic melanoma and were rendered free of detectable disease after surgical resection. All patients received six immunizations, except for 1 patient (no. 16) in the 30- μ g dose group who developed recurrent disease after four vaccinations (<4 weeks) and was removed from protocol. Toxicity was restricted to local erythema and induration at vaccination sites lasting 2–5 days in all patients, and flu-like symptoms lasting 1–3 days,

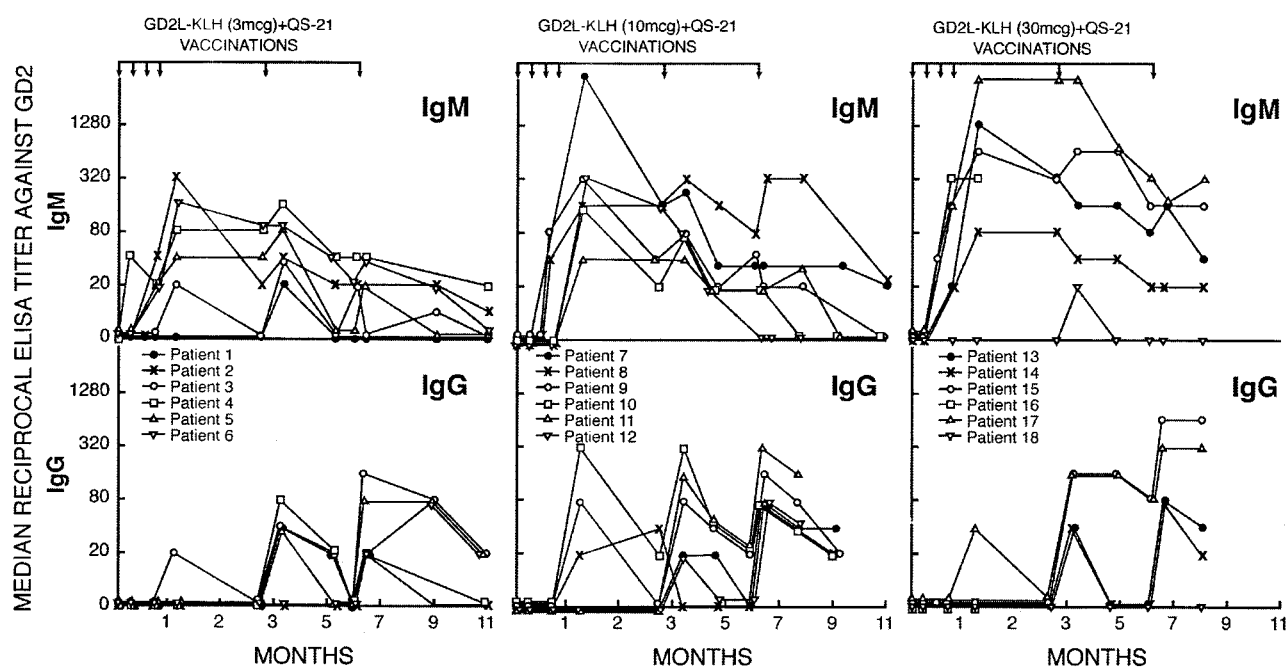


Fig. 2 ELISA titers against GD2 after immunization of patients with different doses of GD2L in a GD2L-KLH conjugate plus QS-21 vaccine.

which were grade 1 after 64 of the 107 immunizations and grade 2 after 5 of the 106 immunizations. No grade 3 or 4 toxicity was noted. This is the pattern of side effects previously associated with administration of several different vaccines containing 100 μ g of QS-21 (14, 15, 17, 21, 22).

Detecting Antibodies against GD2 ELISA. IgM and IgG titers against GD2 before, during, and after immunizations for all 18 patients are shown in Fig. 2 and summarized in Table 1. IgM and IgG antibody responses were induced in most patients. In general, the IgM response peaked after the third immunization, whereas the IgG response peaked after the fourth or fifth (booster) vaccinations. Although there was no significant difference between the antibody responses after the 3-, 10-, or 30- μ g doses, there was a suggestion of increasing ELISA responses with dose escalation.

Cell Surface Reactivity by FACS and CDC. Vaccine-induced IgM antibodies reacted with the tumor cell surface as demonstrated by FACS (Table 1 and Fig. 3). Reactivity was strongest at the 30- μ g dose level in which 5 of 6 patients demonstrated at least a 5-fold increase in percent-positive cells and at least a doubling in the median fluorescent index. At the 30- μ g dose, only sera from patient 18 (which had demonstrated no GD2 reactivity by ELISA) failed to show cell surface reactivity. Reactivity of vaccine-induced IgG antibodies against GD2 on the tumor cell surface could not be demonstrated in any patient. CDC was strongest and most consistent in patients treated at the 30- μ g dose. Patient 18 was the only patient at the 30- μ g dose level who failed to respond to vaccination. This prompted a review of concomitant medications taken by all 18 patients. Patient 18 was the only one who received anti-inflammatory medication (100 mg of celecoxib twice daily) throughout the course of treatment and testing.

Specificity Analysis of the Antibody Response by ITLC.

Sera from the 6 patients vaccinated at the 30- μ g dose level were analyzed by ITLC, as demonstrated for patient no. 17 in Fig. 4 and summarized in Table 1. No patient had reactivity with GD2 before vaccination. Sera from all but patient no. 18 demonstrated potent reactivity against GD2 ganglioside prepared from bovine GD1b or extracted from tumor biopsy specimens. Postimmunization sera from patient 17 reacted primarily with GD2 but also with GM2. Sera from patients 13, 14, 15, and 16 reacted strongly with GD2 but did not react with purified GD3, GM2, or GM3 or against these and other gangliosides present in the tumor extract.

DISCUSSION

Compared with GM2, GD2 is a weak immunogen. As shown previously, among 26 melanoma patients vaccinated with irradiated melanoma cells that expressed comparable amounts of GM2 and GD2, 12 patients developed antibodies against GM2, whereas only two developed antibodies against GD2 (23). In subsequent studies, immunization with a GM2-KLH conjugate plus immunological adjuvant QS-21 resulted in anti-GM2 antibodies by ELISA in virtually 100% of patients and reactivity with tumor cells expressing GM2 by FACS in 90% of patients (14, 15), confirming that antibodies against GM2 are readily inducible. Most recently, we immunized patients with GM2-KLH and GD2-KLH plus QS-21 (24). Although >90% of patients produced high titer antibody responses against GM2, <50% produced antibodies to GD2. Increasing doses of GD2 were tested; 30-, 70-, and 130- μ g doses of GD2 in the conjugate were comparable with each other and superior to 3 and 10 μ g,

Table 1 Antibody response against GD2 after vaccination with increasing doses of GD2L in a GD2L-KLH plus QS-21 vaccine

Patient no. (dose)	ELISA with GD2 Reciprocal peak titer ^a		FACS with NMB-7 % positive cells (MFI) ^b				CDC with NMB-7 % cytotoxicity ^c	ITLC ^d		
	IgM	IgG	IgM		IgG			Pooled gangliosides ^e	Sarcoma extract	0.5 µg GD2
			Pre	Post	Pre	Post				
3 µg										
1	20	20	10 (20)	16 (21)	11 (6)	10 (5)	12			
2	320	0	10 (10)	98 (42)	11 (6)	17 (9)	86			
3	40	160	10 (13)	6 (7)	11 (5)	10 (5)	2			
4	80	80	10 (6)	12 (7)	10 (5)	8 (5)	3			
5	80	80	11 (7)	20 (9)	10 (5)	9 (6)	28			
6	160	80	10 (15)	8 (14)	10 (6)	12 (5)	46			
10 µg										
7	2560	80	10 (95)	38 (205)	10 (10)	9 (10)	70			
8	320	40	10 (91)	15 (119)	10 (7)	7 (7)	40			
9	320	160	10 (43)	53 (113)	10 (7)	9 (8)	98			
10	160	320	9 (60)	13 (72)	12 (7)	18 (9)	0			
11	40	320	10 (66)	21 (65)	9 (7)	8 (6)	0			
12	320	80	10 (52)	73 (150)	9 (7)	13 (8)	10			
30 µg										
13	1280	80	10 (90)	92 (352)	10 (8)	11 (8)	67	+++	+	+++
14	80	80	10 (46)	56 (98)	10 (6)	10 (6)	27	++	+	++
15	640	640	10 (61)	70 (135)	10 (8)	37 (11)	42	+++	++	+++
16 ^f	320	0	10 (63)	50 (132)	10 (8)	16 (14)	32	+++	++	+++
17	2560	160	10 (60)	88 (287)	9 (6)	17 (8)	45	+++	+++	+++
18	20	0	10 (71)	19 (112)	10 (9)	9 (8)	7	-	-	-

^a ELISA titers 1:40 and above are considered positive.

^b mAb 3F8 used as a positive control for FACS and CDC, it showed >90% positive cells and 75% lysis, respectively. MFI, mean fluorescence intensity.

^c Specific chromium release after immunization minus specific chromium release before immunization.

^d Only 30-µg dose tested by ITLC. The TLC immune reaction was graded as - (negative), + (weakly positive), ++ (positive) and +++ (strongly positive).

^e Pooled gangliosides are GD2, GD3, GM2, and GM3.

^f Patient 16 received only four vaccines.

but only 3 of 13 patients treated at the higher doses had antibody titers > 1/160.

In the current study, we have demonstrated that after immunization with GD2L-KLH, 10 of 12 patients (83%) vaccinated at the 10- or 30 µg-dose of GD2L produced antibody titers of $\geq 1/320$. Furthermore, 5 of 6 patients immunized with the 30-µg dose of GD2L had both strong antibody reactivity against cell surface GD2 by flow cytometry and a high level of CDC against GD2-positive tumor cells. In our previous trials with GD2-KLH plus QS-21 vaccines, we never observed anti-GD2 antibodies capable of binding cell surface GD2 and inducing CDC. Therefore, the use of GD2L in place of GD2 in the KLH conjugate has produced a dramatic augmentation of the relevant immunogenicity of GD2. It is interesting to note that the 1 patient at the 30-µg GD2 dose level (patient 18) who did not develop anti-GD2 antibodies was found to have taken 100 mg of celecoxib twice daily throughout the course of vaccinations and testings (a protocol violation).

The basis for the increased immunogenicity of ganglioside lactones remains unclear. Possibilities that have been proposed include: (a) increased rigidity of lactones resulting in a more consistent conformation; (b) the lactone assumes a unique conformation, which is more immunogenic, and the resulting anti-

bodies are able to react with the native structure, and (c) the reduced negative charge of lactones augments immunogenicity. Although the expression of ganglioside lactones had been previously described (25, 26), Nores (18, 27) was the first to suggest the potency of lactones as antigens and immunogens. He demonstrated that immunization of mice with GM3L resulted in significantly more hybridomas against GM3 than was possible by immunization with standard GM3 and hypothesized that GM3L expression on the tumor cell surface was augmented by both the lower pH of many tumors and by the intense expression of GM3 ganglioside on tumor cells. Ding and Magnusson (28) have described additional augmentation of the number of hybridomas resulting from immunization of mice with ganglioside lactams as opposed to lactones. As with the lactones, the lactams demonstrate similar structure and rigidity and have the same reduced negative charge as lactones. The further increase in lactam immunogenicity probably results from increased stability at neutral pH. In either case (*i.e.*, immunization with GM3L or GM3 lactam), many of the resulting hybridoma antibodies were reactive with GM3.

We have now demonstrated similar results after immunization of patients with GD2 lactone. Antibodies produced after immunization with GD2L-KLH plus QS-21 reacted well with

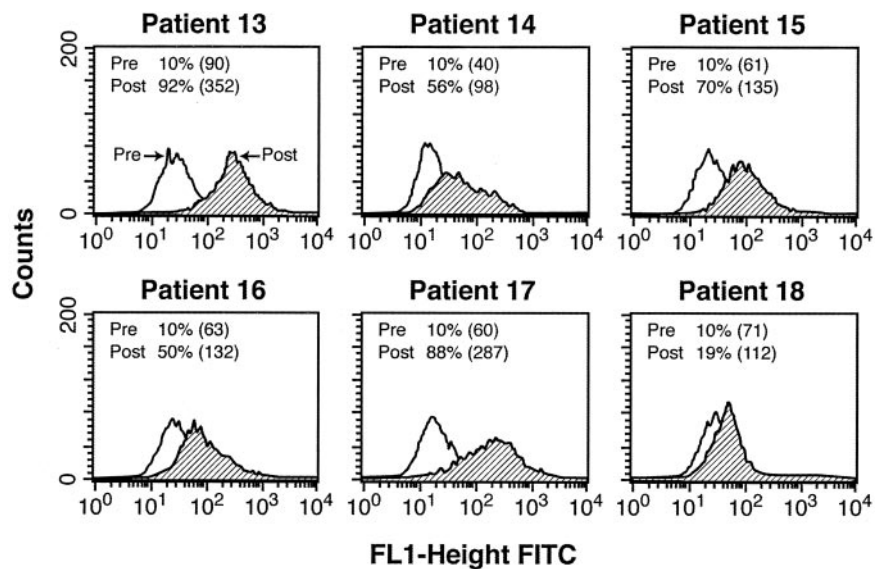


Fig. 3 IgM FACS results before and after immunization with GD2L-KLH plus QS-21 for patients who received a 30- μ g GD2L dose. % Positive cells (Mean Fluorescence Intensity).

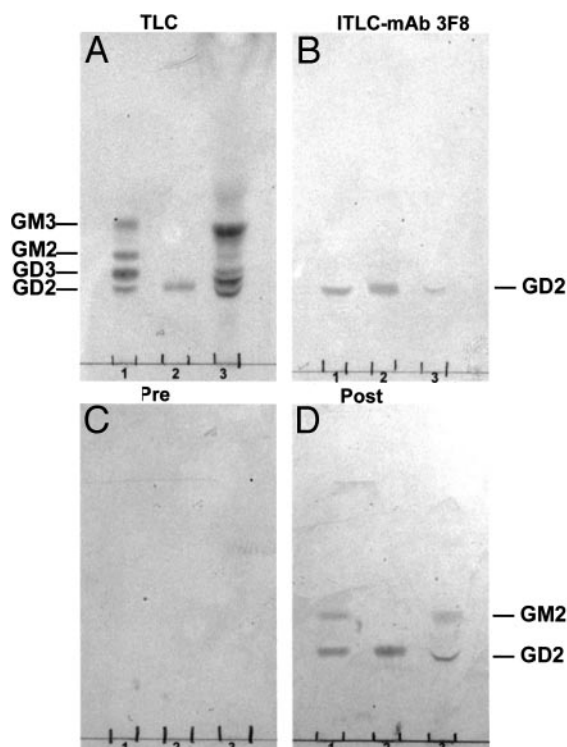


Fig. 4 TLC and ITLC for patient 17 before and after vaccination with GD2L-KLH plus QS-21. Lane 1, GD2, GD3, GM2, and GM3; Lane 2, GD2; and Lane 3, sarcoma extract. A, TLC was sprayed with 0.2% resorcinol and heated at 100°C for 15 min; B, ITLC with mAb 3F8 at 1:500 dilution; C, ITLC with preserum (patient no. 17) at 1:50 dilution; and D, ITLC with postserum (patient no. 17) at 1:50 dilution.

GD2. As originally proposed by Nores for GM3, we have demonstrated that GD2L is significantly more immunogenic than the native GD2 structure and can function as a potent immunogen for inducing antibody responses against the native

GD2 structure. It is interesting to note that this is distinct from our previous experience with GD3 congeners, where immunization with GD3 amide plus *Bacillus Calmette-Guérin* resulted in antibody titers $\geq 1/160$ in all immunized patients, but these antibodies failed to react with GD3 or tumor cells expressing GD3 (29). The use of GD3L plus *Bacillus Calmette-Guérin* in that setting resulted in a low-level antibody response against GD3L that could not be demonstrated to react with wild-type GD3 or tumor cells expressing GD3. We demonstrated subsequently that generation of an antibody response against GD3 required both the use of GD3L as antigen and conjugation to KLH plus the use of immunological adjuvant QS-21; conclusions consistent with the results presented here for GD2 (17).

Despite the relatively high antibody titers against GD2 induced by the GD2L-KLH plus QS-21 vaccine and the duration of these high titer antibodies for at least 6 months in the majority of patients immunized at the 10- and 30- μ g dose levels, no evidence for autoimmunity was seen. GD2 is known to be expressed in the brain, on peripheral sensory nerves, and on a subpopulation of B lymphocytes (7). Administration of mAbs against GD2 (4, 5) did not result in central nervous system toxicity (presumably as a consequence of the blood brain barrier). However, treatment of patients with high doses of some anti-GD2 mAbs has resulted in significant peripheral neuropathies in occasional patients (5), although treatment with other anti-GD2 mAbs has not (4). Interestingly, all anti-GD2 mAbs cause profound pain during the period of antibody administration and for several hours afterward. This is presumably a consequence of GD2 expression on peripheral sensory nerve fibers, but it is paradoxical that the pain lasts only several hours with no sequelae, whereas antibody titers of the administered mAbs remain high for days or weeks. None of the patients immunized with GD2L-KLH plus QS-21 experienced a pain syndrome, presumably because of the relatively low levels of circulating anti-GD2 antibodies compared with patients infused with anti-GD2 mAb (23, 24).

These results form a basis for using GD2L-KLH plus

QS-21 vaccine in larger clinical trials in the future. However, GM2, GD2, and GD3 are differentiation antigens present on melanomas, sarcomas, and neuroblastomas as a consequence of their normal cells of origin rather than their necessity for the malignant phenotype. Consequently, treatment with vaccines against all three of these gangliosides will probably be required to increase the total level of cell surface bound antibodies and limit escape by tumor cells that fail to express any one of three. On the basis of these results, we plan to incorporate the GD2L-KLH vaccine into a trivalent vaccine, including GM2-KLH and GD3L-KLH plus QS-21 in the near future for testing in patients with melanoma, sarcoma, and neuroblastoma.

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