Vaccines containing purified GM2 ganglioside elicit GM2 antibodies in melanoma patients

(cell-surface antigens/cancer vaccines/immunotherapy)

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ABSTRACT GM2, GD2, and GD3 gangliosides are expressed on the surface of human melanoma cells and represent potential targets for immunological control of melanoma growth by monoclonal antibodies and active immunization. The immunogenicity of GM2 was investigated by analyzing the humoral immune response of melanoma patients to vaccination with cell lines selected for high GM2 expression and with vaccines containing purified GM2. The whole-cell vaccine and vaccines containing purified GM2 and bacillus Calmette-Guérin (BCG) elicited GM2 antibody in a high proportion of patients, particularly in GM2/BCG-vaccinated patients pretreated with cyclophosphamide and given a GM2/BCG booster immunization. Vaccines containing purified GM2 and *Salmonella minnesota* R595 as the adjuvant were also effective, but only in patients pretreated with cyclophosphamide. GM2 antibodies in vaccinated patients were of the IgM class and were cytotoxic for GM2-positive targets in the presence of human complement.

Gangliosides are prominent cell-surface constituents of melanoma and other tumors of neuroectodermal origin. Three gangliosides, the monosialoganglioside GM2 and the disialogangliosides GD2 and GD3, are of particular interest to tumor immunologists because of their potential as targets for passive immunization with monoclonal antibodies (mAbs) and for active immunization with cancer vaccines. Despite the presence of GM2, GD2, and GD3 in normal brain and other tissues (1), these gangliosides are immunogenic in mice and humans; mouse mAbs have been generated against GM2 (2), GD2 (3, 4), and GD3 (5-8), and human sera and human mAbs with reactivity for GM2 (9-12), GD2 (11, 13, 14), and GD3 (15) have been identified.

Over the past decade we have immunized sequential groups of melanoma patients with a variety of melanoma cell vaccines (16-20). These vaccine trials were based on our serological analysis of the humoral immune response of melanoma patients to cell-surface antigens of autologous and allogeneic melanomas (21), and each vaccine was constructed to contain melanoma surface antigens known to be immunogenic in humans. Although vaccinated patients readily produced antibody to HLA-related alloantigens and heterologous serum components in the vaccine, only rarely was antibody elicited to more restricted melanoma antigens, such as class 1 (unique), or GD2 or other class 2 (shared) melanoma antigens. In parallel vaccine studies in the mouse, we have identified immunizing procedures that facilitate the serological response to tumor antigens (22-24). In the case of GM2, immunization with GM2-expressing tumor cells or purified GM2 only infrequently induced GM2 antibody in mice, whereas vaccines containing GM2 with adjuvants such

as bacillus Calmette-Guérin (BCG) or *Salmonella minnesota* R595 were far more effective (24).

In the present study, we have examined the immunogenicity of GM2-containing vaccines in stage III melanoma patients. Two types of vaccines were used: a whole-cell vaccine containing high levels of GM2 and vaccines containing purified GM2 with or without microbial adjuvants.

METHODS

Patients. For the studies involving vaccination with purified GM2, patients with AJCC stage III melanoma (i.e., metastases restricted to regional skin and lymph nodes) were considered eligible if tumors and regional lymph nodes had been resected within 4 months and if they were free of detectable melanoma. The studies with the whole-cell vaccines involved patients with Clark's level IV or V primary melanoma or palpable regional lymph node metastases, who were scheduled for regional lymph node dissection. In these cases, the initial vaccine was administered at least 10 days prior to surgery. None of the patients had received prior chemotherapy or radiation therapy. Patients were examined at 6-week intervals. Chest x-rays, liver function tests, and urinalysis were performed at 3-month intervals. Blood for serologic tests was obtained at 2-week intervals.

Gangliosides. GM2 was prepared by treating GM1 with β -galactosidase (G. W. Jourdan, Michigan State University, Ann Arbor, MI) according to published methods (14). GM1, GD1a, GD1b, and GT1 were purchased from Supelco (Bellafonte, PA). GD2 was generously provided by Herbert Wiegandt (University of Marburg, Federal Republic of Germany). Ganglioside extraction, identification, and quantification were performed as described (2).

Serological Procedures. Typing of cell lines for expression of cell-surface gangliosides with mAbs 5-3, 3F8, and R24 was performed as described (2, 3, 5). The enzyme-linked immunosorbent assay (ELISA) (2) was performed with rabbit anti-human IgM, anti-human IgG, or protein A conjugated to alkaline phosphatase (Zymed Laboratories, San Francisco). Antibody titer was defined as the highest serum dilution yielding an OD ≥ 0.190 . Complement-dependent cytotoxicity assays (22) were performed with normal human serum (diluted 1:3) as the complement source. Reagents for ITLC (Gelman) (2) were peroxidase-conjugated goat anti-human IgM and goat anti-human IgG (Tago, Burlingame, CA) diluted 1:500.

Whole-Cell Vaccines. Procedures used for establishing human astrocytoma cell line SK-MG-14 and human melanoma cell line SK-MEL-31 have been described (25). The JB-RH mouse melanoma cell line was established by J. Berkelhammer *et al.* (26) and a subclone, JB-RH-16, selected

Table 1. GM2, GD2, and GD3 composition of whole-cell vaccine

	Cell line		
	JB-RH-16	SK-MG-14	SK-MEL-31
Cell-surface expression of ganglioside*			
mAb 5-3 (α GM2)	65,000	65,000	22,000
mAb 3F8 (α GD2)	0	25,000	2.5×10^6
mAb R24 (α GD3)	0	0	1,000
Ganglioside content of cell line†			
GM2	12.4	4.5	6.9
GD2	0	5.4	2.0
GD3	0	2.1	1.0
Ganglioside content of combined vaccine‡			
GM2	173.8		
GD2	54.3		
GD3	22.7		

*Antibody titer (reciprocal).

†Micrograms per 10^7 cells.

‡Micrograms per 2.2×10^8 cells.

for high GM2 expression, was established in our laboratory (2). Methods for culturing and harvesting cells for vaccine production have been described (19). Cells were irradiated with 10,000 rads (1 rad = 0.01 gray) from a ^{60}Co source and

frozen viable in dimethyl sulfoxide. On the day of vaccination, cells from each line were thawed rapidly, washed three times in phosphate-buffered saline (PBS), pooled in roughly equal numbers, and injected. The median total number of cells per vaccine was 2.2×10^8 suspended in 1 ml of PBS, with $\approx 40\%$ viability (as judged by trypan blue exclusion). No bacterial adjuvant was used. Two or three vaccinations were administered at 5-day intervals immediately prior to lymph node dissection; two or three additional vaccinations were given at 4-week intervals beginning 4 weeks after surgery for a total of five vaccinations. The vaccine was administered intradermally on a rotating basis involving all extremities.

Purified GM2 Vaccines. To prepare GM2 vaccines without adjuvants, 100 μg of GM2 was dissolved in 1 ml of PBS. For vaccines containing BCG, 10^7 viable units of BCG (Tice strain, University of Illinois), or 3×10^6 units in the case of patients showing strong reactions to BCG, were suspended in distilled water by sonication and added to tubes containing 100 μg of dried GM2. The suspension was lyophilized and suspended in PBS shortly before vaccine administration. *S. minnesota* mutant R595 (kindly provided by Jerry McGhee, University of Alabama) was boiled in 1% acetic acid for 1 hr as described (27), washed, dried, and stored frozen. For vaccine preparation, 0.5 mg of R595 was suspended in PBS by sonication and added to GM2 in the same manner as

FIG. 1. GM2 antibody response of stage III melanoma patients after immunization with a whole-cell vaccine or purified GM2 ganglioside vaccines. Each curve represents the response of an individual patient. Arrows indicate time of Cy injection or vaccine injection. The adjuvant used in the booster vaccine was the same as the adjuvant used in the initial vaccines, except in cases in which BCG was replaced by R595.

described for BCG. Patients immunized with GM2 vaccines received four vaccinations intradermally at 2-week intervals on a rotating basis to uninvolved extremities. In addition, some patients immunized with GM2 vaccines containing BCG or R595 received a booster immunization between 3 and 5 months after the fourth vaccination.

RESULTS

Vaccine Characteristics and Serological Response of Vaccinated Patients. Table 1 summarizes the characteristics of the whole-cell vaccine constructed from three cell lines; a mouse melanoma cell line and melanoma and astrocytoma cell lines of human origin. These cell lines were selected for high surface expression of GM2, as indicated by reactivity with a mouse mAb detecting GM2 (2). Five vaccines containing purified GM2 were tested, one with GM2 alone and four with BCG or R595 as adjuvants. In two of these trials, patients were pretreated with low-dose cyclophosphamide (Cy) (200 mg/m²) 3 days prior to the initial vaccination. The whole-cell vaccine, though containing no bacterial adjuvant, resulted in induration and erythema (>8 cm in diameter at 48 hr) in 5 of 6 patients and low-grade fever (<39°C) in 4 patients. Four patients experienced tenderness and swelling in the draining lymph nodes and 5 of the 6 patients showed prominent hyperplasia in the resected lymph nodes. These reactions in skin and lymph nodes, which increased with each vaccination, were not seen in our previous trials with human melanoma cell vaccines. It seems likely, therefore, that this heightened inflammatory reaction is attributable to an anti-mouse response. Vaccination with GM2 alone or GM2/R595 was well tolerated; no side effects were detected. GM2/BCG vaccines resulted in low-grade fever (<39°C) and marked local ulceration in 5 of 11 patients, requiring a decrease in the BCG dose (3 × 10⁶ organisms) or use of R595 in place of BCG for the booster vaccination. No neurologic or other detectable abnormalities were associated with GM2 vaccination.

Fig. 1 and Table 2 show the results of ELISAs for GM2 antibody in serum from normal individuals and from nonvaccinated and vaccinated melanoma patients. The frequency and titer of GM2 antibody in normal individuals and nonvaccinated melanoma patients were similar; ≈80% were negative and only one normal individual had a titer above 1:40. The whole-cell vaccine induced GM2 antibody in high titer (1:80 or greater) in 5 of 6 vaccinated patients. No GM2 antibody was induced in patients immunized with GM2 alone. Addition of BCG to the purified GM2 vaccine resulted in GM2

FIG. 2. Detection of GM2 antibody in sera from vaccinated melanoma patients by immunostaining. Identical TLC plates were stained with resorcinol (far left) or were allowed to react with patients' sera. A, ganglioside standards. B and C, sera from two patients vaccinated with GM2/BCG: GM2 antibody titers by ELISA 1:160 and 1:320. D and E, sera from two patients treated with Cy and vaccinated with GM2/BCG: GM2 antibody titers by ELISA 1:80 and 1:160. F, serum from a patient vaccinated with GM2/R595: GM2 antibody titer by ELISA 1:20.

antibody production, particularly in patients pretreated with Cy or given a booster immunization 12–16 weeks after the last vaccine injection. The effect of Cy was also evident in the case of GM2 vaccines with R595 as the adjuvant; 2 of 6 patients pretreated with Cy produced GM2 antibody, whereas no GM2 antibody was detected in patients not treated with Cy. R595, in contrast to BCG, was not effective as an adjuvant in booster immunizations. No increase in GM2 titers was found in 4 Cy-treated patients given the GM2/R595 vaccine and booster immunizations with GM2/R595 (14 weeks after the last vaccine injection). Because of increasing local inflammatory and systemic reactions induced by BCG in some patients, 5 of the 11 patients initially vaccinated with GM2/BCG vaccine received booster injections of a GM2/R595 vaccine. Four of the 6 patients given the GM2/BCG booster immunization showed a strong rise in GM2 titer; no increase in GM2 titers was found in the 5 patients given the GM2/R595 booster immunization.

Specificity Analysis of Sera from Vaccinated Patients. Sera from the 19 patients with anti-GM2 titers of 1:40 or greater were tested for reactivity with GD1a, GM1, GD2, GD3, and GM3 by ELISA and ITLC. Reactivity was restricted to GM2, with the exception of serum from 1 patient in the whole cell vaccine trial that recognized GD2 at a titer of 1:40. All sera with a titer of 1:80 or higher were also analyzed by ITLC. Reactivity was restricted to GM2. Fig. 2 shows ITLC tests with four sera having an anti-GM2 titer of 1:80 or higher by ELISA. Sera with lower anti-GM2 titers could not generally

Table 2. GM2 antibody titers (ELISA) of normal individuals, untreated melanoma patients, and melanoma patients after immunization with a whole-cell vaccine or purified GM2 vaccines

Treatment	Total no. of patients	No. of patients with a given titer (reciprocal)						Statistical significance,* P
		0	20	40	80	160	320	
Untreated								
Normal individuals	44	37	4	2	1	0	0	
Stage III melanoma patients†	50	37	8	3	0	0	0	
Vaccinated with whole cells‡								
SK-MEL-31, SK-MG-14, and JB-RH-16	6	1	0	0	3	2	0	<0.001
Vaccinated with purified GM2‡								
GM2 alone	5	4	1	0	0	0	0	1.000
GM2/R595	5	3	2	0	0	0	0	1.000
Cy + GM2/R595§	6	0	1	3	0	2	0	0.009
GM2/BCG§	5	0	1	1	1	1	1	<0.001
Cy + GM2/BCG§	6	0	1	0	2	2	1	<0.001

*Fisher's exact test of number of vaccinated patients with titers ≥1:80 compared with 92 controls.

†Serum from 2 patients excluded from evaluation because of nonspecific reactivity (refs. 11, 24).

‡Peak titer observed after vaccination.

§Including booster vaccine.

Table 3. GM2 antibodies in sera of vaccinated melanoma patients: Comparison of titers determined by ELISA and cytotoxic tests with human complement

GM2 antibody titer by ELISA (reciprocal)	No. of patients	Median OD (range) at 1:40 serum dilution	Median cytotoxicity titer (range)	
			20% lysis end point	50% lysis end point
0	8	0.04 (0.01–0.06)	ND	ND
20	6	0.12 (0.08–0.23)	ND	ND
40	4	0.25 (0.21–0.35)	20 (5–80)	0 (0–5)
80	6	0.40 (0.38–0.84)	80 (20–80)	5 (0–20)
160	7	0.66 (0.41–1.45)	160 (80–320)	20 (10–80)
320	2	1.42 (1.40, 1.45)	320 (320)	30 (20, 40)

ND, not determined.

be analyzed by ITLC. GM2 antibodies in vaccinated patients belonged to the IgM class; tests with an IgG indicator system revealed no IgG anti-GM2.

Complement-Dependent Cytotoxicity of GM2 Antibodies. Sera from patients developing high titers of GM2 antibody after vaccination were found to be cytotoxic for GM2-positive target cells in the presence of normal human serum as complement source. GM2-negative target cells were not lysed. Table 3 shows the relation between anti-GM2 titers detected by ELISAs and cytotoxicity tests (with SK-MG-6 astrocytoma cells, ref. 2). A positive correlation was seen between antibody titers in both assays.

DISCUSSION

The identification of melanoma cell-surface antigens that are immunogenic in the host of origin has been the object of our analysis of sera (21), cytotoxic T cells (28, 29), and mAbs (15, 30) derived from melanoma patients. Three general categories of melanoma cell-surface antigens that are immunogenic or potentially immunogenic in the autologous host have been defined; these range from highly restricted antigens that are detected only on autologous melanoma cells [class 1 (unique) antigens], to antigens present on a subset of melanomas as well as a limited range of other cell types (class 2 antigens), to antigens that are widely distributed on melanomas and other cell types (class 3 antigens) (21). Biochemical characterization of these melanoma antigens is limited, but class 1 (unique) antigens appear to be glycoproteins (31) and one of the best-studied class 2 melanoma antigens is the ganglioside GD2 (13, 14). As reactivity against these antigens is found in only a small percentage of melanoma patients, we have attempted to induce antibodies to class 1 or class 2 antigens using vaccines of irradiated cells expressing these antigens (16–20). These human trials have not been successful and prompted us to define conditions required for a consistent humoral immune response to tumor antigens in the mouse, including the class 1 antigen of Meth A sarcoma (22, 23) and the ganglioside GM2 (20, 24). Adjuvants and pretreatment with low doses of Cy were important factors in the mouse studies, and results of the present human trials indicate their importance in melanoma patients.

Irie, Tai, Morton and colleagues have also identified the immunogenicity of GM2 in their studies of melanoma patients (11, 12). They isolated stable cultures of Epstein-Barr virus-transformed B cells from a melanoma patient that produced a mAb to GM2 (12). In addition, Tai *et al.* have immunized melanoma patients with melanoma cell vaccines containing a mixture of gangliosides and found that reactivity against GM2 was induced in 10/26 patients (11). As in the present study, reactivity against GD2 was only rarely detected (2/26 patients) in their series and no antibody against GD3 or GM3 was found.

We and others have detected low levels of GM2 antibody in some normal individuals (10) and nonvaccinated stage III

melanoma patients. Natural growth of melanoma in the skin and regional lymph nodes does not appear to be a potent stimulus for generating GM2 antibody, since antibody levels in stage III melanoma patients are no higher than in normal individuals. The fact that most melanoma patients can be induced to develop high levels of GM2 antibody after vaccination is surprising in view of the presence of these gangliosides in brain and other tissues of neuroectodermal origin. The other finding of importance for further clinical trials of GM2 vaccines is that high titers of GM2 antibody had no demonstrable ill effect on these patients.

There is a suggestion from the present study that melanoma recurrence is delayed in patients developing GM2 antibody. Evaluation of 31 vaccinated patients in this study observed for 15 months showed that 5 of 14 patients with GM2 titers $\leq 1:20$ are disease free, as compared to 14 of 17 patients with GM2 titers $\geq 1:40$. The two groups did not differ in known prognostic indicators. However, conclusions about the value of GM2 vaccines and the relation between levels of GM2 antibody and melanoma recurrence require a randomized controlled study involving larger numbers of patients. Nonetheless, the generation of cytotoxic antibodies to a cell-surface antigen expressed on the surface of melanoma cells after immunization with a pure antigen is an important step in realizing the goals of a melanoma vaccine. The IgM response to the GM2 vaccine is relatively short-lived; methods to prolong the response and to induce IgG and T-cell responses to GM2 are challenges that need to be addressed. In addition, the precise distribution of GM2 in normal tissues needs to be assessed, as does the frequency of GM2 in primary and metastatic melanoma. Finally, it will be important to determine the melanoma GM2 phenotype in individual patients before and (in the case of recurrence) after GM2 vaccination, to establish whether GM2 phenotype correlates with anti-GM2 response and whether immunoselection of GM2-negative variants occurs.

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