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Antibody response after immunization with the gangliosides GM1, GM2, GM3, GD2 and GD3 in the mouse

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Summary. The gangliosides GM2, GD2 and GD3 are differentiation antigens expressed on the cell surface of human melanomas and other cancers of neuroectodermal origin. We have compared the antibody response after vaccination with gangliosides GM1, GM2, GM3, GD2 and GD3 in the mouse. Purified gangliosides were injected subcutaneously alone or attached to *Salmonella minnesota* mutant R595 after pretreatment of the mice with low-dose cyclophosphamide. Spontaneous GM1 antibodies, but not antibodies against the other gangliosides, were detected in many mice, the incidence increasing with age. Purified gangliosides injected alone (in saline) induced no antibody response. R595/GM1 and R595/GD3 vaccination induced consistent high-titer antibody responses. R595/GM2 and R595/GD2 induced occasional antibody responses, and R595/GM3 induced no antibody response. Comparison of the antibody responses induced against these gangliosides in the mouse with those in man reveals that GM1, GM3 and GD2 have a similar immunogenicity in both species while the relative immunogenicity of GM2 and GD3 is reversed. To understand better the basis for these differences, the antibody responses against the five gangliosides in man and the mouse were compared with their known expression in normal tissues. No correlation was detected between ganglioside expression in normal brain and immunogenicity, consistent with this being a cloistered site. The antibody responses did correlate inversely with expression in normal non-brain human and murine tissues. Variations between species of ganglioside immunogenicity may reflect variations in ganglioside expression in normal tissues.

Introduction

A variety of gangliosides are expressed on the cell surface of human malignant melanomas. While none are tumor-specific, the distribution on normal tissues of several gangliosides is highly restricted. The importance of these gangliosides as potential targets for active specific immunotherapy with tumor vaccines has been suggested by regression of melanoma and neuroblastoma metastases in some patients treated with anti-GD3 and anti-GD2 mouse monoclonal antibodies [7, 18]. Although GD3 and GM3 are the most prevalent gangliosides on human melanomas, they are not good immunogens, no antibodies against them have been detected in human sera. Many high-titer sera reactive with GM2 or GD2 have been detected, generally after immunization with irradiated melanoma cells that contain ten times more GD3 and GM3 than GD2 and GM2 [25, 27, 44], further emphasizing the poor immunogenicity of GD3 and GM3 in man. GM1 is not detectable on most melanomas. Intentional immunization with GM1 has not been attempted, though GM1 can be assumed to be quite immunogenic in man, on the basis of GM1 antibodies detected in the sera of some normal donors [11, 40] and patients with multiple sclerosis, lupus erythematosis, strokes and head trauma [11]. There is little direct evidence on the relative immunogenicity of these gangliosides in mice. If the number of laboratories developing murine monoclonal antibodies can be used as a gauge of ganglioside immunogenicity, then GD3 [5, 9, 17, 36, 49] and GD2 (4, 6, 17, 20, 49] appear to be the most immunogenic followed by GM2 [30, 33] GM3 [45] and GM1 [28].

We have recently reported that immunization with GM2 coated on *Salmonella minnesota* mutant R595 in mice pretreated with low-dose cyclophosphamide (to reduce suppressor cell activity) was an effective method for inducing a cytotoxic antibody response against melanoma cells expressing GM2 [26]. The studies described here use this approach to determine the relative immunogenicity (in terms of antibody response) of purified GM1, GM2, GM3, GD2 and GD3 gangliosides in the mouse. GD3 and GM1 are identified as most immunogenic. The immunogenicity of these gangliosides in the mouse is also compared with their known immunogenicity in man, and the basis for the clear differences explored.

Materials and methods

Animals. Female Balb/c-C57BL/6 Fl mice, 6 weeks of age, were obtained from the Jackson Laboratory, Bar Harbor, Maine. Immunization was begun within 2 weeks.

Serological assays. Mice were bled from the retro-orbital sinus before and 2 weeks after each vaccination and serum samples for serological testing (approximately 0.1 ml) were stored at −20° C. ELISA were performed using rabbit anti-(mouse IgM) and protein A linked to alkaline phospha-

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Abbreviations used. The abbreviations for gangliosides are according to the system of Svennerholm [43]; ELISA, enzyme-linked immunosorbent assay; R595, *S. minnesota* rought mutant R595

tase (Zymed, San Francisco, Calif) as previously described [33]. The absorbance of samples with pretreatment mouse serum was subtracted from post-treatment values to yield the experimental values at each titer. To eliminate the effect of nonspecific "sticky" sera, sera were also tested on plates which had been processed identically, but to which no ganglioside had been added. The absorbance at each titer obtained on this plate was subtracted from the experimental value, yielding the corrected absorbance. Serological titer in ELISA is defined as the highest dilution yielding a corrected absorbance of 0.10 or greater. All assays were repeated on two or more occasions to ensure consistency. Immune stains (dot blots) were performed as previously described [15, 46] with slight modifications. In brief, 0.25–1 μg ganglioside was spotted on nitrocellulose paper strips. The strips were blocked at room temperature for 2 h in phosphate-buffered saline containing 5% immunoglobulin-free fetal calf serum, 1% bovine serum albumin and 0.05% sodium azide. Serum was diluted 1/150 with the same immunoglobulin-free mixture and the strips were incubated in Accutron trays (Schleicher and Schüll, Keene, NH) at room temperature for 16 h. Strips were washed five times with the phosphate-buffered saline mixture and incubated for 5 h with horseradish-peroxidase-conjugated anti-(mouse IgM) antibody diluted 1:200 (Zymed, San Francisco, Calif). Peroxidase staining was performed as previously described [33]. Stains were quantified as negative, 1+, 2+ or 3+ as shown in Fig. 1.

Gangliosides. GD2 was prepared by treating GD1b with β-galactosidase purchased from Dr. G. W. Jourdian, University of Michigan, Ann Arbor, Michigan. The enzyme treatment was performed essentially as described by Cahan et al. [3]. GM2 was purchased and GD3 was a gift from Fidia Research Labs, Abano Terme, Italy. GM3 was purified from dog erythrocytes as previously described [33]. GM1, GD1a GD1b, and GT1 were purchased from Supelco Inc. (Bellafonte, Penn). Gangliosides used for vaccine production were pure as determined by thin-layer chromatography.

Cyclophosphamide. Cyclophosphamide (Cytoxan, Mead Johnson and Company, Evansville, Ind) was administered at a dose of 15 mg/kg I.p. 3 days before the first vaccination.

Vaccine preparation. Salmonella minnesota mutant R595 was cultured and boiled in 1% acetic acid as previously described [26], washed and stored. The day before vaccination, R595 was resuspended in distilled water by sonication and added to tubes containing dried ganglioside. The R595/ganglioside suspension was lyophilized. Immediately prior to vaccine administration R595/ganglioside or ganglioside alone was resuspended in normal saline.

Administration of vaccines. In each experiment five mice, selected randomly from the same shipment, were immunized with a given vaccine. Vaccinations were administered 3–4 weeks apart subcutaneously in a total volume of 0.1 ml/mouse. Vaccines contained 10, 50 or 150 μg ganglioside (GM1, GM2, GM3, GD2 or GD3), or 150 or 450 μg pooled gangliosides (GM2 plus GD2 plus GD3), alone or with 0.5 mg R595. No toxicity or morbidity was detected as a consequence of vaccine administration.

Results

Mice were immunized twice with vaccines containing R595, purified ganglioside or R595 plus various gangliosides. Sera were obtained before immunization and at regular intervals after vaccination, and tested by ELISA and dot-blot assays.

Serological response to vaccines containing a single ganglioside (GM1, GM2, GM3, GD2 or GD3)

Sera from unimmunized 2–3-month-old mice and from mice immunized with R595 alone showed occasional GM1 reactivity (see below) but no serological reactivity against GM2, GM3, GD2 or GD3. The serological response of mice after immunization as defined by ELISA and immune stains is shown in Table 1. Vaccines containing gangliosides alone were not immunogenic. R595 vaccines containing the higher dose of ganglioside (150 μg) were slightly but not significantly more immunogenic than those containing 50 μg, but comparing the groups of 10–20 mice immunized with each ganglioside, the gangliosides GM1 and GD3 were significantly more immunogenic than the others ($P = 0.01$ by the log rank test). Some antibodies against GM2 and GD2 were detected after immunization. No antibody response against GM3 was detected.

Anti-IgM immune stain results were generally consistent with ELISA results. Antibodies were predominantly IgM though occasional IgG antibodies were identified by ELISA.

Serological response to vaccines containing three gangliosides (GM2, GD2 and GD3)

Ten mice were immunized with R595 plus three gangliosides. The results are shown in Table 2. Once again the immunogenicity of GD3 was significantly greater than that of GM2 or GD2 ($P < 0.05$).

Specificity of reactive sera defined by dot-blot immune stains

Sera from all mice were analyzed for reactivity against GM1, GM2, GM3, GD2 and GD3 in immune stains using

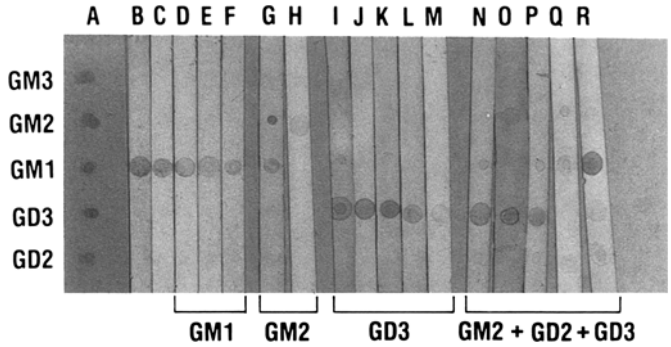

Fig. 1. Ganglioside standards were applied to silica gel strips and stained with resorcinol (A), or to nitrocellulose strips and allowed to react with sera from individual mice (B–R). A, ganglioside standards. B and C, normal 1-year-old mice: reactions on GM1 graded 3+ and 2+. D–F, GM1-immunized (12-week-old) mice: GM1 1+(3). G and H, GM2-immunized mice: GM2 2+, 1+; GM1, 2+. I–M, GD3-immunized mice: GD3 3+(3), 2+,1+. N–R, mice immunized with GM2, GD2 and GD3: GD3 3+(3), 1+; GM2 2+, 1+(3); GD2 1+(4); GM1 3+, 2+, 1+(2)

Table 1. Antibody reactivity by ELISA and immune stain after two immunizations with vaccines containing a single ganglioside (GM1, GM2, GM3, GD2 or GD3)

Vaccine	Ganglioside dose (μg)	No. of mice	Anti-IgM[a]		Anti-IgG[a]	
			ELISA titer	Immune stain	ELISA titer	Immune stain
GM1	50	5	20, 20			
GM2	50	5	20, 20			
GM3	50	5				
GD3	50	5				
R595	0	10				
R595/GM1	10	5	20 (3), 40			
	50	5	40 (2), 320 (2), 640	2+ (2), 3+ (2)		
	150	5	80, 160, 320 (2)	1+, 2+ (2), 3+ (2)		
R595/GM2	10	5	20			
	50	10	40 (4), 80	1+		
	150	5	160, 40	1+, 2+	80	
R595/GM3	10	5				
	50	5			20, 20	
	150	5	40, 40			
R595/GD2	50	10	20, 20		40	
	150	5	20, 40		40, 80, 80	
R595/GD3	10	10	40 (2), 20 (4)	1+, 1+	20, 40, 640	
	50	10	80 (2), 160 (5), 320 (2)	1+, 2+ (4), 3+ (3)	20, 40, 640	2+
	150	5	80, 320 (3)	2+, 3+ (3)	40, 80	

[a] Tested on immunizing ganglioside, or (in the case of the R595 alone vaccine) on GM2 and GD3. Blank, no reactivity

Table 2. Antibody reactivity by ELISA and immune stain after two immunizations with vaccines containing R595 and three gangliosides (GM2, GD2 and GD3)

Individual ganglioside dose (μg)	Target ganglioside	Anti-IgM[a]		Anti-IgG[a]	
		ELISA titer	Immune stain	ELISA titer	Immune stain
50	GM2	20			
150	GM2	20 (2)	1+ (2)	80	
50	GD2	40	1+		
150	GD2				
50	GD3	40, 40, 80	1+, 3+		
150	GD3	20, 160, 320 (2)	2+ (2), 3+	20, 80	

[a] Tested on immunizing ganglioside. Blank, no reactivity

anti-IgM second antibody; sera from mice showing IgG ELISA reactivity were analyzed in addition by immune stains with anti-IgG and protein-A-peroxidase conjugates. Results with reactive sera (IgM) from some of the immunized mice are shown in Fig. 1. Except for several sera from unimmunized mice or mice immunized with other gangliosides that were reactive with GM1, reactivity was restricted to the immunizing ganglioside. Specificity analysis of the sera with ELISA IgG reactivity titers of 1/80 or greater was inconclusive, anti-IgG and protein A dot-blot immune stains were generally unreactive.

Several mice, immunized with GM2, GD2 or GD3, R595 alone, or unimmunized, produced antibody reactive with GM1 by ELISA and immune stains (see Fig. 1). These sera were not broadly cross-reactive and increased their reactivity in samples drawn from the same mice months apart. Since GM1 is expressed extensively in brain tissue, we were concerned that the appearance of anti-GM1 antibodies in some mice immunized with other gangliosides was related to the vaccinations. To explore this further, we performed ELISAs and dot blots with sera from normal mice of different ages. Production of IgM anti-GM1 antibodies (but not antibodies against GM2, GM3, GD2 or GD3) increased with age in unimmunized mice: 5/5, 12/16 and 0/5 mice aged 1 year, 6 months and 6 weeks, respectively, showed reactivity titers of 1/80 or greater, which were restricted to GM1. The immunogen inducing these GM1 antibodies is not known. No neurological sequelae resulted.

Discussion

The studies described here are part of our continuing attempts to improve the serological response induced by tumor vaccines in preclinical and clinical trials [22–24, 27]. The serological approach is emphasized because serological techniques are precise and can be used to monitor clinical trials. In previous studies in the mouse, we have tested a variety of immunization approaches and have reported increased serological responses against tumor antigens in-

cluding GM2 after anti-suppressor cell treatment with low-dose cyclophosphamide and the use of vaccines containing endotoxin, lipid A or *s. minnesota* mutant R595 as adjuvants [23, 24, 26]. We show here that this same approach (cyclophosphamide + R595/ganglioside) with five different gangliosides in the mouse results in consistent high-titer antibodies against GM1 and GD3, occasional antibody responses against GM2 and GD2, and no antibodies against GM3. In man, GM1 is known to be immunogenic, GD2 weakly immunogenic and GM3 not immunogenic at all, similar to results in the mouse. However, GM2 antibodies are frequently induced in man [27, 44] while GD3 antibodies have not been detected in human sera. The relative immunogenicity of GD3 and GM2 in man and the mouse is reversed.

To determine whether these differences in antibody induction after immunization with GM1, GM2, GM3, GD2 and GD3 are explained by differences in expression of these gangliosides in normal tissues of the two species, we reviewed previously published studies on ganglioside content of normal tissues defined by biochemical extraction [1, 12–14, 19, 21, 31, 32, 34, 37, 38, 42, 50]. Comparable data for ganglioside expression in five normal human and murine tissues (brain, kidney, liver, spleen and erythrocytes) were available. While we are aware that these may not be representative of all tissues, they are considered the major ganglioside-rich tissues. Ganglioside content (especially GM2 and GM3) is known to vary considerably between different inbred strains [21] so only data in C57Bl/6 × Balb/c (the mice used in these immunization studies) or C57BL/6 or Balb/c mice were considered. To facilitate comparison, the results of this literature review are presented in Table 3. While the highest concentration of most gangliosides was in normal brain [1, 19, 42], no correlation could be detected between antibody titers and ganglioside expression in brain tissue. This is not surprising since it has long been suspected that the brain is an "immunologically privileged site" [16], effectively cloistering its antigens from the immune system. In other tissue [1, 12–14, 31, 32, 34, 37, 38, 50], GM1 expression is very low in both species and GM3 expression very high. Expression of GM2, however, differed greatly between man and the mouse, being very low in man and very high in the mouse. Fewer data are available for GD2 than for other gangliosides but GD2 expression appears to be relatively low in murine and human tissues. GD3 expression was low in murine tissues and intermediate in man. Tissue typing with murine monoclonal antibodies on various normal human tissues has demonstrated high expression of GD2 on sensory peripheral nerve fibers [8] while GD3 has been shown to be expressed on a subpopulation of T cells [48] as well as on cells in several other tissues [39], tissues not studied by extraction procedures. Comparable tissue typing in the mouse has not been conducted. There was, therefore, an inverse correlation between the expression of GM1, GM2, GM3, GD2 and GD3 on normal tissues and their immunogenicity.

The apparent lack of toxicity in mice producing ganglioside antibodies, despite expression of the antigen on various normal tissues, may relate to subthreshhold expression at the cell surface of normal tissues making autoimmune recognition unlikely, as has been theorized with regard to GM3 antibodies [35]. We demonstrate here that though GM1 and GD3 are expressed in large amounts on normal murine brain, they are highly immunogenic when administered with R595. This is consistent with the finding that cloistered antigens may be immunogenic [29, 41]. The lack of neurological sequelae in mice with GM1 or GD3 antibodies may be a consequence of the "blood-brain barrier" which may result, at least in part, from the tight junctions of cerebral capillary endothelia [22] and effectively restrict access of circulating antibodies.

These studies are most consistent with the view that the antibody response against individual gangliosides after vaccination is inversely proportional to the extent of their expression on normal tissues outside the brain. This concept of "horror autotoxicus" dates back to 1900 when it was first expressed by Ehrlich [10]. Our studies also suggest that there is a difference between the level of antigen expression on normal tissues permitting antibody induction after appropriate vaccination, and the level sufficient for induction of autoimmune disease. Since the level of GM2, GD2 and GD3 expressed in human melanoma cells [47] is at least tenfold higher than in the major organ systems

Table 3. Comparison of ganglioside composition of murine and human normal tissues

Tissues	Strain[a]	References	Total lipid-bound sialic acid (μg/g tissue)	GM1	GM2	GM3	GD2	GD3	Others
				(% of total gangliosides)[b]					
Murine									
Brain	B6	19, 42	800	11	–	1		3	15
Kidney	F1	32	11	+	+	++	×	×	
Liver	B6 + Bc	14, 24, 32	67	+	70	20	–	–	10
Spleen	F1	32	32	+	+	++	×	×	
Erythrocytes	B6 + Bc	12, 13	1	–	45	–	–	–	55
Human									
Brain		1	500	18	3	4	5	7	63
Kidney		38	15	–	–	74	–	19	7
Liver		34	20	1	–	91		3	5
Spleen		50	60	1	–	84	–	2	13
Erythrocytes		37, 31	6	–	–	25	–	–	80

[a] Strains: B6, C57BL/6; Bc, Balb/c; F1, C57BL/6 × Balb/c F1. Blank, not tested

[b] –, not detected; +, detected as minor component but not quantified; ++, detected as a major component but not quantified; ×, not tested [32] but detected by TLC and immune staining using monoclonal antibodies 3F8 against GD2 [17] and R24 against GD3 [9] in our laboratory as previously described [21], but not quantified

(excluding brain) that we reviewed, it may be that antibodies induced against these gangliosides will be capable of inducing effective anti-melanoma immunity without inducing autoimmune disease.

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