Induction of IgG Antibodies against G_{D3} Ganglioside in Rabbits by an Anti-idiotypic Monoclonal Antibody

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Abstract

Anti-idiotypic MAb were raised in syngeneic mice against a mouse MAb recognizing G_{D3} ganglioside (MAb R24). Two anti-idiotypic MAb, designated BEC2 and BEC3, recognized distinct determinants on MAb R24 that mapped near or within the G_{D3}-binding site. New Zealand white rabbits, which express G_{D3} on normal tissues, were immunized with either BEC2, BEC3, or control MAb FLOPC-21. All rabbits developed high and equivalent titers of antibodies against mouse immunoglobulins. Immunization with BEC2 and BEC3 induced rabbit antibodies expressing R24 idiotype as demonstrated by their ability to inhibit BEC2 binding to R24. Antibodies (IgG and IgM) reacting with G_{D3} developed in five of eight rabbits immunized with BEC2 but not in rabbits immunized with BEC3 or with control MAb. Serum antibodies against G_{D3} did not cross-react with other gangliosides. These results show that MAb BEC2 can mimic G_{D3} ganglioside and can induce antibodies against G_{D3} ganglioside despite expression of G_{D3} on normal rabbit tissue. (*J. Clin. Invest.* 1991. 88:186–192.) Key words: melanoma • immunization • internal image • glycolipid

Introduction

Gangliosides are polymorphic glycolipids found on surface membranes of most cell types and, as a rule, are weakly immunogenic (1–3). The major antigenic determinants of gangliosides are carbohydrates which typically generate T cell-independent humoral immune responses. However, in certain circumstances, gangliosides can function as autoantigens: (*a*) antibodies against gangliosides have been detected in sera of patients with autoimmune diseases (4); (*b*) antibodies against the gangliosides G_{D2} and G_{M2} have been found in the sera of melanoma patients (5), and human IgM MAb against gangliosides have been isolated from B cells of melanoma patients (6–8); (*c*) immunization with selected purified gangliosides has been shown to induce antibody responses in mice (1); and (*d*) immunization with G_{M2} and BCG adjuvant has induced IgM antibodies against G_{M2} in a majority of patients with metastatic melanoma (9).

G_{D3} is a ganglioside abundantly expressed on most human melanoma cells (10). G_{D3} has received attention as a possible target for active and passive immunotherapy of human melanoma and other neuroectoderm-derived tumors (2, 11–13). In mice, which express little G_{D3} on normal tissues (1), antibodies against G_{D3} can readily be induced by immunization with purified G_{D3} plus adjuvant (1). However, in humans and other mammalian species, G_{D3} is more abundantly expressed on selected normal tissues, including the central nervous system, adrenal medulla, and other tissues derived from the neuroectoderm (14, 15). In these species, immunization with purified G_{D3} plus adjuvant does not generally induce antibodies against G_{D3} (1).

An alternative strategy for inducing antibodies against gangliosides involves the use of anti-idiotypic MAb (16). This approach requires that a polypeptide Ig variable region can mimic a carbohydrate determinant, providing a surrogate immunogen. Anti-idiotypic MAb that mimic other carbohydrate antigens, such as bacterial LPS (17–19) and $\beta 2 \rightarrow 6$ fructosan (20), have been produced. In the LPS system, anti-idiotypic MAb can be superior to the original antigen in priming neonatal mice to respond (17). We describe an anti-idiotypic MAb that mimics antigenic determinants on G_{D3} and induces antibody responses in a species (rabbits) that express G_{D3} on normal tissues.

Methods

Animals. Mice were purchased from Charles River Professional Services, Wilmington, MA and New Zealand White (NZW)[1] rabbits were from Hazelton Research Products (Denver, PA) or from Cocalico Biologics, Inc. (Reamstown, PA).

Materials. Cells were cultured in RPMI 1640 with 10% fetal bovine serum or 10% Serum Plus (Hazelton Research Products, Inc., Lenexa, KS). Media was supplemented with 2 mM glutamine, 1% nonessential amino acids, penicillin (100 U/ml), and streptomycin (100 U/ml).

G_{D3} was purified from bovine brain. Other purified gangliosides were purchased from Calbiochem-Behring Corp., La Jolla, CA.

R24, an IgG3 MAb raised in a [C57BL × BALB/c]F_1 mouse against human melanoma, specifically recognizes the ganglioside G_{D3} (10). V1-R24 is a variant of R24 in which both light chains have been substituted by MOPC-21 light chains derived from the NS-1 myeloma fusion partner. These light chains alter the antigen binding site enough to decrease the avidity for G_{D3} by 40-fold (21). The BALB/c myeloma proteins MOPC-21, MOPC-104E, MOPC-141, MOPC-315, UPC-10, TEPC-15, TEPC-183, Y5606, and FLOPC-21 were purchased from Sigma Chemical Co., St. Louis, MO. MAb 3F8 and 455 were gifts from Dr. Nai-Kong Cheung and Dr. John Mendelsohn, respectively (Memorial Sloan-Kettering Cancer Center, New York). MAb A33, HT29-15, S22, F23, and 34-417 were gifts from Dr. Sydney Welt (Memorial Sloan-Kettering Cancer Center). OKB7 and M195 were gifts from Dr. David Scheinberg (Memorial Sloan-Kettering Cancer Center). MAb

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1. *Abbreviations used in this paper:* NZW, New Zealand white.

2G10 was a gift from Dr. Piergiorgio Natali (Regina Elena Cancer Institute, Rome, Italy). F36/22 was from Dr. T. Ming Chu (Roswell Park Memorial Institute, Buffalo, NY). MAb 6H4 and 14G2a were gifts from Dr. Ralph Reisfeld (Scripps Clinic and Research Foundation, La Jolla, CA). ME361 was a gift from Dr. Meenhard Herlyn (The Wistar Institute, Philadelphia, PA). MAb M111, C350, TA99, C5, and K9 have been previously reported (10, 22). MAb 11A is an anti-G_{D3} MAb recently produced in our laboratory. SK-MEL-256 is a human melanoma cell line that expresses cell surface G_{D3}.

Production of anti-idiotypic MAb against R24. Mice syngeneic to R24 ([C57BL × BALB/c]F_1) were immunized with weekly intraperitoneal injections of R24 (100 μg R24/injection) adhered to fixed *Staphylococcus aureus* cells (Calbiochem-Behring Corp.). After three intraperitoneal immunizations, mice were boosted intravenously with R24 in PBS 1 and 3 wk later. With every immunization, the mice received an i.p. injection of 1 μg recombinant human IL-1β (a gift from Robert C. Newton, DuPont Glenolden Lab, Glenolden, PA). 3 d after the last boost, splenocytes were collected and fused with SP2/0 mouse myeloma cells using standard fusion techniques. Selected hybridomas (see below) were subcloned by limiting dilution and grown as ascites-producing tumors in syngeneic mice. Anti-idiotypic MAb were purified either by protein A or protein G affinity chromatography.

ELISA. To measure anti-idiotypic reactivity, R24 F(ab')$_2$ fragments, prepared as previously described by pepsin digestion (23), were diluted in 0.05 M borate, pH 8.6, and adsorbed to 96-well plates (1 μg/well) overnight at 4°C. After blocking with 5% nonfat milk, material to be tested for anti-idiotypic reactivity was diluted in PBS/1% BSA (wt/vol)/0.05% polyoxyethylene sorbitan monolaurate (Tween 20) (vol/vol) and incubated for 1 h at room temperature. Bound antibody was detected using an alkaline phosphatase-conjugated second antibody specific for the Fc portion of mouse IgG (Cappel Organon Teknika Corporation, West Chester, PA). Plates were developed with *p*-nitrophenyl phosphate substrate in a diethanolamine buffer and absorbance monitored at 405 or 410 nm using an ELISA plate reader.

The specificity of both anti-idiotypic MAb was tested by inhibition assay in which purified anti-idiotypic MAb was preincubated with serial dilutions of inhibiting MAb for 1 h. The mixture was then added to 96-well plates coated with R24 F(ab')$_2$ fragments and assayed by ELISA as above. The concentration of inhibiting MAb that inhibited 50% of anti-idiotypic MAb binding to R24 F(ab')$_2$ fragments (IC_{50}) was calculated and compared with the IC_{50} of R24 assayed as a positive control. An MAb was considered to be inhibitory if the MAb IC_{50}:R24 IC_{50} ratio was less than 10. To map binding sites, cross-blocking experiments were performed using anti-idiotypic MAb biotinylated using either biotin hydrazide (24) or *N*-hydroxysuccinimidobiotin (both from Sigma Chemical Co.). Nonbiotinylated, blocking anti-idiotypic MAb was added to 96-well plates coated with R24 (1 μg/well). After 1 h, biotinylated anti-idiotypic MAb was then added. Plates were washed after 1 h and wells were incubated with alkaline phosphatase-conjugated avidin for 15 min. Plates were developed as above.

The expression of R24 idiotype by rabbit antibodies was measured by inhibition assay in which various dilutions of rabbit sera were preincubated with biotinylated BEC2 (0.38 μg/ml) and the mixture was added to 96-well plates coated with R24 (1 μg/well). After 1 h, wells were washed and bound BEC2 was detected by adding alkaline phosphatase-conjugated avidin and developed as above.

To measure anti-G_{D3} reactivity in the serum of immunized rabbits, purified G_{D3} was adsorbed to 96-well plates (100 ng/well). After blocking wells with 5% nonfat milk, test serum was diluted in PBS/1% BSA/0.05% Tween 20 and added to wells for 1 h. Plates were washed and bound rabbit antibody was detected using alkaline phosphatase-conjugated goat antiserum specific for rabbit IgG (Sigma Chemical Co.). Anti-G_{D3} serum titers were defined as the highest serum dilution yielding an absorbance reading greater than three standard deviations above the mean background reading. Rabbits were considered to have developed anti-G_{D3} antibodies if a fourfold or greater rise in titer above preimmune serum was observed in serum samples taken from at least two separate time points.

Rabbit anti-mouse antibodies were measured by adding test serum to 96-well plates coated with mouse IgG (Sigma Chemical Co.) (0.5 μg/well). Bound antibody was detected with alkaline phosphatase-conjugated goat anti-rabbit IgG as above.

Immunizations with anti-idiotypic MAb. Rabbits received five injections of MAb (100 μg/injection) at intervals of at least 3 wk over the course of 3 mo. For the initial immunization, MAb was mixed with Freund's complete adjuvant and administered subcutaneously at multiple sites. The second and third injections were also given subcutaneously but with Freund's incomplete adjuvant. For the final two injections, MAb was injected intramuscularly without adjuvant.

Inhibition of R24 binding to melanoma cells. R24, diluted in PBS with 5% gamma globulin-free FCS, was preincubated with various dilutions of anti-idiotypic MAb. After 1 h, each mixture was plated onto SK-MEL-256 cells growing in 60 × 10 Nunclon microtiter plates (Nunc, Roskilde, Denmark). Plates were incubated 1 h and then washed with PBS/2% gamma globulin-free FCS. Protein A-conjugated human erythrocytes were added for 45 min as previously described (22). The plates were washed again and the percentage of target cells rosetted was scored by phase microscopy. Wells in which more than 20% of target cells were rosetted were considered positive.

Immunoblotting. Reactivity of rabbit anti-G_{D3} antibodies against various gangliosides was measured by immunoblotting. Purified ganglioside was applied to nitrocellulose strips (Schleicher & Schuell, Inc., Keene, NH) as distinct dots (1 μg/dot) and allowed to dry. Strips were blocked with PBS/1% BSA/0.05% Tween 20 or with 5% nonfat milk. The strips were then incubated overnight in a 1:10 dilution of test serum. After washing, bound rabbit IgG was detected by incubating nitrocellulose strips with ^{125}I-protein A (New England Nuclear, Boston, MA) and exposing strips to Kodak X-Omat film (Eastman Kodak Co., Rochester, NY). To detect bound IgM, strips were incubated with peroxidase-conjugated goat anti-rabbit IgM (Cappel Organon Teknika) and developed with the chromogen 4-chloronaphthol.

Results

Anti-idiotypic MAb against R24. Syngeneic mice were immunized with MAb R24 (as described in Methods). Three mice demonstrating the highest titer serum reactivity against R24 F(ab')$_2$ fragments were selected for fusions. 651 wells were screened from three separate fusions. Two hybridomas were identified and subcloned that produced MAb recognizing F(ab')$_2$ fragments of R24. The hybridomas and the MAb produced by them were designated BEC2 (IgG2b) and BEC3 (IgG3).

The specificities of BEC2 and BEC3 were determined by inhibition assays using a panel of MAb (Table I). MAb BEC2 recognized determinants expressed on the anti-G_{D3} MAb C5 (IgG3) and 11A (IgM) as demonstrated by IC_{50}:R24 IC_{50} ratios of less than 10. BEC2 did not specifically recognize any other MAb in the panel that included other MAb against G_{D3} and MAb against other gangliosides. These results demonstrated that BEC2 recognized an idiotope expressed on some anti-G_{D3} MAb but not all. BEC2 did not recognize V1-R24, a low avidity variant of R24 containing R24 heavy chains and MOPC-21 light chains, which implies that the idiotope recognized by BEC2 requires specific light chain domains. BEC3 appeared to recognize a slightly different idiotope, which was present both on R24 and on V1-R24, because both of these MAb inhibited binding of BEC3 to R24 F(ab')$_2$ fragments. However, the IC_{50} for V1-R24 was approximately ninefold higher than the IC_{50} for R24. This result implies that BEC3 recognizes an idiotope on R24 that is also present on V1-R24, perhaps in a different conformation, resulting in a ninefold lower binding affinity.

Table I. Specificity of BEC2 and BEC3 Anti-idiotypic MAb as Measured by Inhibition of Binding to R24

	Inhibiting MAb		Inhibition (MAb IC_{50}:R24 IC_{50} ratio)	
	Isotype	Specificity	BEC2	BEC3
MOPC104E	μ	α 1 → 3 dextrans	−(20.2)	−(10)
11A	μ	G_{D3} ganglioside	+(2.4)	NT
K9	μ	G_{D3} ganglioside	—	NT
TEPC183	μ	ND	−(45.1)	−(17.5)
MOPC315	α	Dinitrophenol	—	—
TEPC15	α	Phosphoryl choline	—	—
MOPC21	γ_1	ND	—	—
HT29-15	γ_1	Adenocarcinoma	—	—
455	γ_1	EGF receptor	—	—
M111	γ_1	gp110	−(29.4)	—
S22	γ_1	ND	—	NT
C350	γ_1	gp180	—	—
14G2a	γ_{2a}	G_{D2} ganglioside	—	—
UPC10	γ_{2a}	β 2 → 6 fructosan	—	—
TA99	γ_{2a}	gp75	—	—
A33	γ_{2a}	ND	—	—
M195	γ_{2a}	CD33	—	—
2G10	γ_{2a}	gp75	NT	—
F23	γ_{2a}	CD13	—	—
ME361	γ_{2a}	G_{D2}/G_{D3} gangliosides	—	—
MOPC141	γ_{2b}	ND	—	—
OKB7	γ_{2b}	CD21	−(29.4)	NT
FLOPC21	γ_3	ND	—	—
Y5606	γ_3	AMP/purines	—	—
F36/22	γ_3	Breast carcinoma	—	—
34-417	γ_3	Sialylated Lewis A	—	—
3F8	γ_3	G_{D2} ganglioside	—	—
6H4	γ_3	G_{D3} ganglioside	−(>66.7)	—
C5	γ_3	G_{D3} ganglioside	+(2.9)	+(1.7)
V1-R24*	γ_3	G_{D3} ganglioside	−(40.8)	+(9.2)

Specificity was assessed by the ability of inhibiting MAb to block binding of BEC2 or BEC3 to R24 $F(ab')_2$ fragments. In general, a three- to sixfold excess of R24 concentration was sufficient for 50% inhibition (IC_{50}). The numbers listed in parentheses represent the ratios of the IC_{50} observed for the inhibiting MAb divided by the IC_{50} for R24. For MAb tested multiple times, the mean value is listed. The absence of an IC_{50} ratio denotes that less than 50% inhibition was observed at all concentrations tested and an IC_{50} for that MAb could not be calculated. *NT*, not tested; *EGF*, epidermal growth factor. * A variant of R24 containing two MOPC-21 light chains derived from the NS-1 myeloma fusion partner. V1-R24 has 40-fold lower avidity for G_{D3} compared with R24.

The determinants recognized by BEC2 and BEC3 were further defined by experiments that showed both BEC2 and BEC3 could cross-inhibit binding of each other to R24 (Fig. 1). BEC2 efficiently inhibited BEC3 binding to R24 while BEC3 was less efficient at inhibiting BEC2 binding to R24. It may be that the idiotopes recognized by BEC2 and BEC3 only partially overlap. Alternatively, BEC2 may bind to R24 with much higher affinity than does BEC3. In experiments to further characterize the R24 idiotopes recognized by BEC2 and BEC3, we found that binding of R24 to purified G_{D3} or to a G_{D3}^+ melanoma cell line was blocked by preincubation with either BEC2 or BEC3.

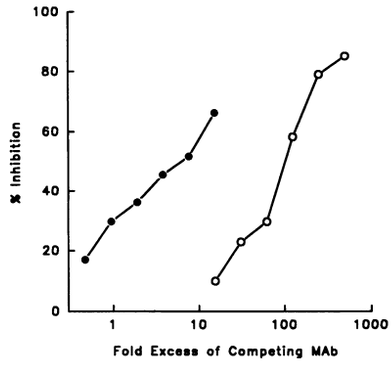


Figure 1. Cross-inhibition of BEC2 and BEC3 binding to R24. Various concentrations of BEC2 (● —— ●) or BEC3 (○ —— ○) were added to 96-well plates coated with R24 (1 μg/well). Biotinylated BEC2 (0.13 μg/ml) was added to wells blocked with BEC3, while biotinylated BEC3 (33 μg/ml) was added to wells blocked with BEC2. Bound anti-idiotypic MAb was detected using alkaline phosphatase-conjugated avidin as described in Methods. Percentage inhibition was calculated by comparison with uninhibited wells.

Fig. 2 shows a representative experiment testing the ability of BEC2 and BEC3 to inhibit binding of R24 to the G_{D3}^+ melanoma cell line SK-MEL-256. Both BEC2 and BEC3 could inhibit R24 binding, and 50% inhibition of R24 binding could be achieved with either a 60-fold excess of BEC2 or a 100-fold excess of BEC3. Similar results were obtained in ELISA assays measuring the ability of BEC2 or BEC3 to block binding of R24 to purified G_{D3} (data not shown). The concentration of anti-idiotypic MAb required for inhibition of R24 binding to G_{D3} implies the relative avidity of R24 for G_{D3} is higher than the avidity of either anti-idiotypic MAb for R24.

Taken together, these results show that BEC2 and BEC3 are anti-idiotypic MAb against R24. Differences in specificity show that BEC2 and BEC3 recognize different idiotopes on R24, although both idiotopes are located near the G_{D3}-binding site of R24.

BEC2 but not BEC3 carries the internal image of G_{D3}. NZW rabbits were chosen to test whether BEC2 or BEC3 carried an internal image of G_{D3} ganglioside. Rabbits provide a reasonable model for immunization of humans based on the following features: (*a*) mouse MAb are xenogeneic both in rabbits and humans; (*b*) purified G_{D3} is not immunogenic in either

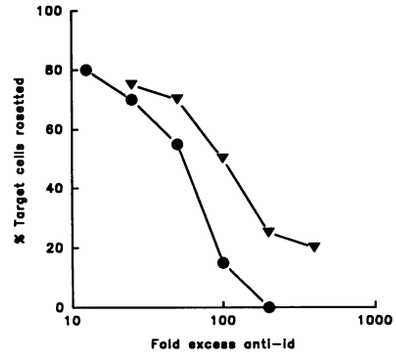


Figure 2. Inhibition of R24 binding to melanoma cells by anti-idiotypic MAb. R24 was inhibited with various concentrations of either BEC2 (— ● —) or BEC (— ▼ —) and then plated onto SK-MEL-256 melanoma cells. Bound R24 was detected using a mixed hemadsorption assay (see Methods). The data are expressed as the mean percentage of target cells rosetted in duplicate wells (y axis) versus the fold excess of inhibiting anti-idiotypic MAb (x axis). For BEC2, the final R24 concentration was 2.5 μg/ml and uninhibited control wells showed 95% rosetting. For BEC3, the final R24 concentration was 1.25 μg/ml and uninhibited wells showed 85% rosetting. *Anti-id*, anti-idiotypic.

rabbits (3) or humans (1); and (*c*) both rabbit (25–27) and human normal tissue (14, 15, 23) can express G_{D3} ganglioside. Immunostaining with MAb R24 confirmed that G_{D3} is expressed on normal rabbit tissues in a distribution similar to human tissues (Cordon-Cardo, C., P. B. Chapman, and A. N. Houghton, unpublished observations).

NZW rabbits were immunized with either BEC2, BEC3, or control MAb (FLOPC-21). As expected, all rabbits developed high titer IgG against mouse Ig after a single inoculation with each of these mouse MAb. Peak anti–mouse Ig titers developed after a median of three (range, two to five) inoculations with a median peak titer of 1:656,000 (range, 1:121,000 to 1:2,000,000). There were no significant differences between the titers of anti–mouse Ig induced by each of the three mouse MAb, demonstrating that the three MAb were equally immunogenic.

Sera were assayed for IgG antibodies against G_{D3} ganglioside. Five of eight rabbits immunized with BEC2 developed IgG antibodies reacting with purified G_{D3} by ELISA, but no antibodies reacting with G_{D3} were induced in six rabbits immunized with either BEC3 or with FLOPC-21 ($P = 0.028$, Fisher's exact probability test). Fig. 3 illustrates the mean preimmunization and peak postimmunization IgG reactivity against G_{D3} for all animals immunized. Immunization with BEC2 induced IgG against G_{D3} with a mean peak titer of 1:160 (range, 1:80 to 1:640) (Fig. 3 *A*). In contrast, after immunization with either BEC3 or FLOPC-21, no detectable reactivity against G_{D3} was induced in any rabbit (Fig. 3, *B* and *C*).

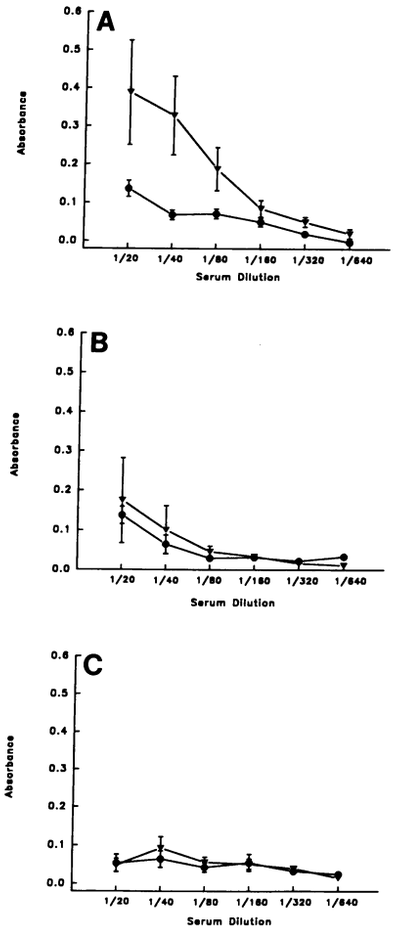


Figure 3. Reactivity against G_{D3} of sera from rabbits immunized with anti-idiotypic or control MAb. Anti-G_{D3} reactivity was measured by ELISA (see Methods) and data are expressed as the mean absorbance of preimmune serum (— ● —) and immune serum showing maximum anti-G_{D3} reactivity (— ▼ —). Error bars represent standard error of the mean. (*A*) Mean reactivity against G_{D3} of sera from eight rabbits immunized with BEC2. (*B*) Mean reactivity against G_{D3} of sera from three rabbits immunized with BEC3. (*C*) Mean reactivity against G_{D3} of sera from three rabbits immunized with FLOPC-21.

We confirmed that the antibody response to G_{D3} was IgG class. First, the antibody response against purified G_{D3} could be detected using ^{125}I-protein A (see Fig. 5 *B* below). Because rabbit IgG, but not IgM, binds protein A (28), this reactivity is presumably due to IgG. Second, G_{D3}-reactive rabbit antiserum was subjected to 45% ammonium sulfate precipitation and separation by size-exclusion chromatography using a FPLC Superose 6 column (Pharmacia Fine Chemicals, Piscataway, NJ). Antibody reactivity against purified G_{D3} eluted in a molecular mass range of ~ 150,000 D, within the range of monomeric IgG but not pentameric IgM (data not shown). Since IgM anti-G_{D3} could be detected in whole serum by immunoblot (see below), it is likely that IgM was not efficiently precipitated or that IgM anti-G_{D3} was inactivated after ammonium sulfate precipitation.

Fig. 4 shows the typical time course of the IgG response against G_{D3} in a rabbit immunized with BEC2. IgG antibodies against G_{D3} were generated after the second, third, and fourth immunizations with BEC2. In some rabbits, titers appeared to decline after the fifth immunization. It should be noted that the last two immunizations were administered without adjuvant. However, IgG antibodies against G_{D3} could be detected as long as 107 d after the last immunization (data not shown), demonstrating that the anti-G_{D3} IgG response could be long lived. While sera with anti-G_{D3} reactivity could inhibit R24 binding to G_{D3}, this effect was not specific. Sera from rabbits immunized with BEC3 or with FLOPC-21 could also inhibit R24 binding to G_{D3} suggesting that this effect was due to rabbit anti-mouse antibodies.

It is not uncommon for antibody responses against gangliosides to cross-react with carbohydrate determinants on distinct gangliosides (6). For this reason, we were interested in analyzing the specificity of the anti-G_{D3} reactivity induced by BEC2. Immunoblot analysis of sera from selected rabbits immunized with BEC2 showed the presence of both IgM (Fig. 5 *A*) and IgG antibodies (Fig. 5 *B*) against G_{D3}. These antibodies did not cross-react with the other gangliosides tested, including the ganglioside G_{M3}, which differs from G_{D3} by the absence of a single sialic acid moiety. Overall, these data demonstrate that, although immunization with BEC2 and BEC3 MAb induced high and equivalent titers of anti–mouse IgG, only immunization with BEC2 induced an IgG response against G_{D3}.

Expression of R24 idiotype by Ab3. In order to determine whether rabbit antibodies induced by BEC2 expressed an R24

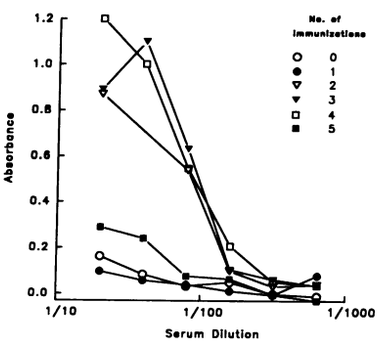
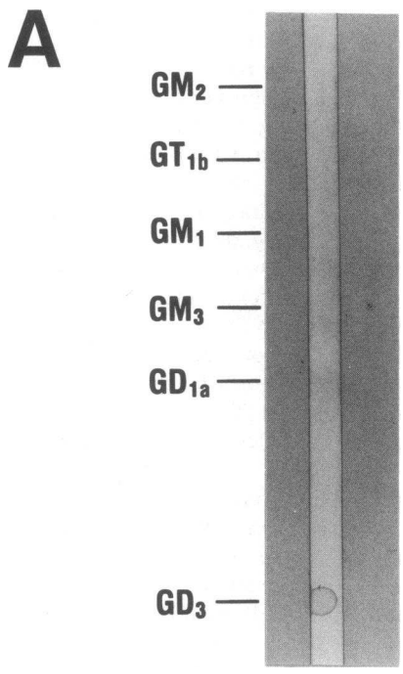


Figure 4. Time course of the IgG response against G_{D3} in a representative rabbit immunized with BEC2. Immunizations were administered on days 1, 17, 31, 57, and 85. Sera were collected preimmunization, and on days 17, 31, 43, 67, and 95. Anti-G_{D3} reactivity was measured by ELISA and expressed as absorbance at 410 nm.



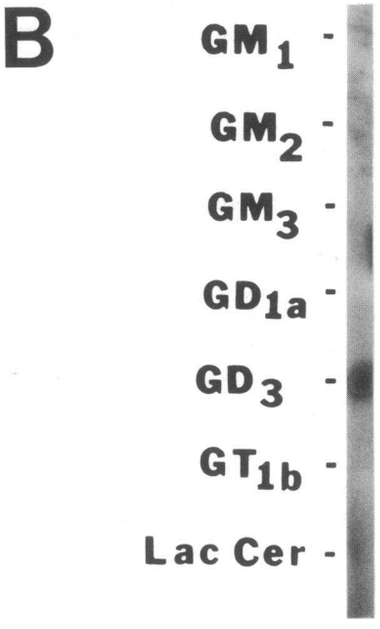


Figure 5. Specificity of antibodies against G_{D3} induced by BEC2. After immunization with BEC2, selected rabbit sera showing anti-G_{D3} reactivity by ELISA was diluted 1:10 and reacted with various purified gangliosides dried on nitrocellulose filter strips. (*A*) Bound IgM was detected using peroxidase-conjugated goat anti-rabbit IgM and (*B*) bound IgG was detected using ^{125}I-protein A. *Lac*Cer, lactosylceramide.

idiotype, the ability of rabbit antisera to inhibit binding between BEC2 and R24 was tested (Fig. 6). All eight rabbits immunized with BEC2 developed antibodies that could inhibit BEC2 binding to R24. These inhibitory antibodies were induced even in the rabbits that did not develop anti-G_{D3} reactivity. Rabbits immunized with BEC3 also developed antibodies capable of inhibiting BEC2 binding to R24, but the titer of these antibodies was considerably lower. Immunization with FLOPC-21 did not induce antibodies capable of inhibiting BEC2-R24 binding, despite the fact that FLOPC-21 induced a comparable level of rabbit anti-mouse antibodies. These data show that immunization with BEC2, and to a lesser extent BEC3, induces antibodies in rabbits that express the R24 idiotype.

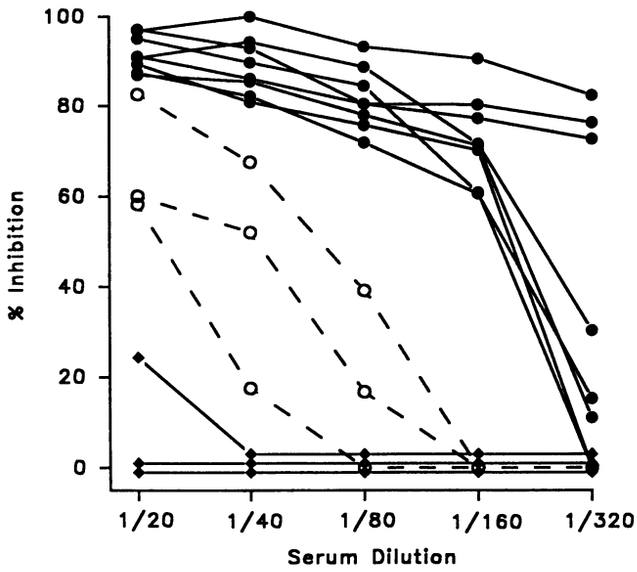


Figure 6. Expression of R24 idiotype by rabbit antibodies induced after immunization with BEC2, BEC3, or FLOPC-21. Dilutions of rabbit sera were preincubated with biotinylated BEC2 (final concentration, 0.38 μg/ml) before being added to 96-well plates coated with R24 (1 μg/well). Bound BEC2 was detected using alkaline phosphatase-conjugated avidin. Data is expressed as the percentage inhibition of BEC2 binding. Sera was obtained from rabbits immunized with BEC2 (— ● —), BEC3 (— ○ —), or FLOPC-21 (— ♦ —). No inhibition was observed with preimmune rabbit sera.

In summary, these data show that BEC2 carries an internal image of G_{D3} ganglioside, based on the finding that immunization of NZW rabbits with BEC2 induced IgG responses against G_{D3} ganglioside. This anti-G_{D3} response occurred despite the expression of G_{D3} by normal rabbit tissues. It is interesting to note that none of the rabbits showed clinical or pathological evidence of adverse effects due to immunization with BEC2. Necropsy specimens of selected rabbits with IgG antibody response against G_{D3} after immunization with BEC2 showed no histologic evidence of immune cell infiltration or tissue destruction in organs that express G_{D3}, such as brain, adrenal gland, liver, and small intestines.

Discussion

Jerne proposed that the immune system can be regulated through a network of antibody/anti-idiotype interactions (29). One of the implications of this model is the existence of anti-idiotypic antibodies that carry internal images of every recognizable determinant. Consistent with this model, anti-idiotypic MAb can be identified that carry the internal image of nonprotein determinants (17–20, 30). In tumor antigen systems, anti-idiotypic MAb have usually been used to mimic protein antigens (16, 31, 32) although Viale and co-workers have described an anti-idiotypic MAb that carries the internal image of an undefined saccharide determinant expressed on tumor-associated glycolipids and glycoproteins (33). We present data that the anti-idiotypic MAb BEC2 carries an internal image of the ganglioside G_{D3}. Presumably BEC2 mimics a determinant that includes a terminal *N*-acetyl neuraminic acid because this moiety is critical for R24 binding to G_{D3} (34).

A number of investigators have shown that anti-idiotypic MAb can be used as surrogate antigens to induce immunity (16). In infectious disease models, there is evidence that selected anti-idiotypic MAb can be better immunogens compared to the original antigen (17). Previous work has shown that purified G_{D3} is a poor immunogen in species that express G_{D3} on normal tissues (1–3), possibly due to a state of relative B cell tolerance to cell surface G_{D3}. The experiments with BEC2 immunization demonstrate that B cells that can secrete antibodies against G_{D3} remain in the immune repertoire of the rabbit.

Titers of anti-G_{D3} antibodies induced by BEC2 were relatively low. Immunoglobulin genes in reactive B cells may have undergone appropriate rearrangement of variable, diversity, and junctional regions, but not somatic mutation necessary for production of high affinity antibodies. The question remains whether these B cells could undergo appropriate and selective somatic mutation, a step presumably requiring T cell help. The ability of BEC2 to induce an IgG response suggests recruitment of T cell help. This may be analogous to a hapten-carrier protein model in which T cells recognizing peptide determinants within the carrier protein (e.g., murine-specific determinants on BEC2) provide help for the B cell population specific for hapten (e.g., the BEC2 idiotope that mimics G_{D3}).

There appeared to be limitations to the IgG response induced by immunization with BEC2 using this route, schedule, and adjuvant. Rabbits generating IgG antibodies against G_{D3} did not show histological evidence of inflammation in G^+_{D3} tissues. It is possible that the affinity and titer of these anti-G_{D3} antibodies were too low or that immunization with BEC2 selected IgG antibodies with little or no effector functions. Although a majority of rabbits immunized with BEC2 developed IgG against G_{D3}, there was a subset of rabbits in which the anti-G_{D3} response was too weak to be scored as positive. We presume that the lack of response in this subset was related to an inability of BEC2 to stimulate the appropriate B cell clones.

It will be important to determine whether BEC2 or other anti-idiotypic MAb can elicit delayed-type hypersensitivity to carbohydrate antigens, which are classically T cell independent. However, it is not clear how carbohydrate antigens could be recognized by T cells, because T cell receptor binding appears to require presentation of antigen by MHC molecules, and binding of carbohydrates to MHC has not been demonstrated. Further studies will be necessary to test whether the immunogenicity of BEC2 can be augmented by evaluating different schedules and routes of immunization, adjuvants, and immune modulators such as interleukins and cyclophosphamide.

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