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Lewis rats immunized with GM1 ganglioside do not develop peripheral neuropathy

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Abstract

Elevated levels of anti-GM1 antibodies are associated with motor nerve syndromes. Although there is a lot of circumstantial evidence that anti-GM1 antibodies may be causing the disease, their precise role remains unclear. In order to study the role of anti-GM1 antibodies in the pathogenesis of peripheral neuropathy, eight Lewis rats were injected with GM1 ganglioside mixed with keyhole limpet hemocyanin (KLH) and emulsified with Freund's adjuvant and three rats were immunized with GM1 in liposomes. Although IgM class anti-GM1 antibodies were detected in all animals immunized with GM1, none of the animals exhibited overt signs of neuropathy during six months after initial immunization. IgG antibody to GM1 was not produced in any of the animals. There was no pathological evidence of nerve damage. These studies suggest that elevated levels of IgM anti-GM1 antibodies by themselves do not cause nerve damage in rats.

Keywords

Motor neuropathy syndromes; antibodies; GM1 ganglioside

1. Introduction

Elevated levels of autoantibodies to glycolipids including gangliosides are frequently found in peripheral neuropathies (Ilyas et al., 1984, 1988b, 2007; Latov, 1995; Quarles and Weiss, 1999; Willison and Yuki, 2002). Monosialoganglioside GM1 is a major glycosphingolipid of both the central and peripheral nervous system. Elevated levels of anti-GM1 antibodies are often associated with motor nerve syndromes. IgG anti-GM1 antibodies are associated with acute motor neuropathy syndromes including acute motor axonal neuropathy variant of the Guillain-Barré syndrome (GBS) (Ilyas et al., 1992b, 2001; Nobile-Orazio et al., 1992; van den Berg et al., 1992; Kornberg et al., 1994) and monoclonal or polyclonal IgM anti-GM1 antibodies are associated with chronic motor neuropathy syndromes (Freddo et al., 1986; Ilyas et al., 1988a; Pestronk et al., 1988, 1990; Baba et al., 1989; Kinsella et al., 1994; Taylor et al., 1996).

The close association of anti-GM1 antibodies with motor neuropathy syndromes lends strong support to the notion that anti-GM1 antibodies play a crucial role in the pathogenesis of

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neuropathy. This notion is supported by the induction of anti-GM1 induces neuropathy in Japanese white rabbits (Yuki et al., 2001). However, the role of anti-GM1 antibodies in the pathogenesis of neuropathy is questioned because passive transfer studies fail to transfer the disease (Hadden et al., 2001; Sheikh et al., 2004; Yuki et al., 2004) and other studies failed to induce neuropathy by active immunization of rabbits with purified GM1 (Lopez et al., 2002; Dasgupta and Yu, 2004). In this study, we immunize Lewis rats with purified GM1 ganglioside. Our data show that although IgM anti-GM1 antibodies were induced in all rats immunized with GM1, none of the animals with elevated IgM anti-GM1 antibodies exhibited overt neurological signs of neuropathy and/or neuropathological changes in peripheral nerves.

2. Methods and Materials

2.1. Reagents

Bovine brain ganglioside mixture and GM1, GD1b, and galactocerebroside were purchased from Sigma, St. Louis, MO. The glycolipids were checked for purity by thin-layer chromatography. Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem, San Diego, CA. Complete and incomplete Freund's adjuvant were obtained from Difco Laboratories, Detroit, MI. Peroxidase-conjugated goat anti-rat IgG or IgM antibodies were purchased from Accurate Chemical and Scientific, Westbury, NY.

2.2. GM1-Liposome preparation

Liposomes were prepared as described (Freimer et al., 1993). Lipid A, DMPC (dimyristoyl phosphatidylcholine), cholesterol and glycolipid antigen were dissolved in chloroform. The solvent was removed using a rotary evaporator. The mixture is then dried in a desiccator for 1 hr. After drying, glass beads (0.5mm) and sterile water were added and the flask was vortexed vigorously to disperse all lipid in the form of liposomes.

2.3. Animal immunization

Female Lewis rats weighing between 170 and 200 g were purchased from Charles River Laboratories. Eight rats were injected subcutaneously at multiple sites in the back (thoracic region) with 200 micrograms of purified GM1 ganglioside mixed with keyhole limpet hemocyanin (KLH) (2 mg/ml) and emulsified with equal volume of complete Freund's adjuvant (CFA). The rats were given booster injections every four weeks for five months with 100 µg GM1 mixed with KLH in incomplete Freund's adjuvant (IFA). Three rats were injected subcutaneously at multiple sites in the back with liposomes containing 100 µg of GM1 (Freimer et al., 1993) and then were given booster injections every four weeks for five months with liposomes containing 100 µg GM1. Four control rats were injected with KLH mixed with CFA only and two control rats were injected with liposomes without GM1 and then boosted every four weeks with KLH emulsified with IFA or liposomes. The animals were examined regularly for clinical symptoms and weighed weekly. Animals were bled from the tail vein prior to initial immunization and then every 4 weeks thereafter.

2.4. Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed essentially as described previously (Ilyas et al., 1984). 100 nanograms of purified GM1 in 20 µl methanol were added to wells in Linbro ELISA plates and the solution was dried by evaporation. The plates were blocked with 1% BSA in PBS at room temperature for 2h. The wells were emptied and 100 µl of rat serum diluted 1:100 in 1% BSA/PBS was added and the plates were kept at 4 °C for 5 h. The wells were washed with cold PBS and 100 µl of peroxidase-conjugated goat anti-rat IgG or IgM diluted in 1% BSA/PBS was added. The plates were then incubated overnight at 4 °C. After washing, 200 µl of substrate solution containing 0.1% o-phenylenediamine in 0.1 M citrate buffer, pH 4.5 and 0.012% H₂O₂ was

added to each well and the plates were incubated for 30 min at room temperature. The absorbance at 490 nm of each well was read on EL 309 Microplate Autoreader. Absorbance values were corrected by subtracting optical densities obtained in wells without antigen. Positive sera were further tested by serial twofold dilutions and the serum titer is given as the highest dilution that showed an absorbance value of 0.1 or greater.

2.5. Thin-layer chromatography (TLC)-immunostaining

Gangliosides were separated on aluminum-backed TLC plates as described previously (Ilyas et al., 2002). The plates were developed in chloroform:methanol:0.2% KCl (50:40:10, v/v/v). The glycolipids were detected by orcinol spray (0.2% orcinol in 2N H₂SO₄). The binding of anti-ganglioside antibodies on TLC was determined as described below. After separating the lipids, the TLC plate was dipped in n-hexane containing 0.1% polyisobutylmethacrylate for 30s and then dried. The plates were then overlaid with 1% BSA in PBS for 2 h in the cold and then with rat serum (1:100 dilution) for 4 h at 4 °C. The plates were washed with cold PBS and then overlaid with peroxidase-conjugated anti-rat IgM (μ-chain-specific) antibodies and kept at 4 °C overnight. The plates were then washed and developed with o-phenylenediamine.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)/Western blotting

Rat brain myelin was prepared as described (Norton and Poduslo, 1973). The SDS-PAGE and Western blotting was carried out in 12.5% polyacrylamide gels using slab electrophoretic cell (Mini-PROTEIN II, Bio-Rad) essentially as described (Ilyas et al., 1992a) except that blots were incubated with rat sera followed by peroxidase-conjugated goat anti-rat IgG or IgM antibody. Peroxidase activity was detected using 3,3'-diaminobenzidine as substrate.

2.7. Pathological examination

The animals were anesthetized with sodium pentobarbital (50 mg/kg) intraperitoneally and perfused through the left cardiac ventricle with 4% paraformaldehyde in PBS (Ilyas et al., 2002). The sciatic nerves and two dorsal ganglia were removed from both sides. The spinal cord with roots was dissected. The tissues were immersed in 3.5% glutaraldehyde in cacodylate buffer, pH 7.4, for an additional 8 h. The tissues were then postfixed in 1% osmium tetroxide for 1.5 h, dehydrated in graded ethanol, and embedded in Spurr's resin. One-micrometer thick sections were stained with toluidine blue for pathological examination with a light microscope.

3. Results

All eight rats immunized with GM1 ganglioside mixed with KLH and emulsified in complete Freund's adjuvant and all three rats immunized with GM1-containing liposomes produced IgM antibodies against GM1. It is of interest that none of the immunized rats produced anti-GM1 IgG antibody after repeated immunization. Serum anti-GM1 IgM antibody titers in immunized rats ranged from 400 to 3,200 (Table 1). The anti-GM1 IgM titers achieved in rats are less than in some patients with neuropathy. Anti-GM1 IgM titers of more than 1:10,000 have been reported in some patients with multifocal motor neuropathy (Pestronk et al., 1990; Kinsella et al., 1994; Taylor et al., 1996) and in patients with neuropathy associated with IgM gammopathy (Pestronk et al., 1990; Kinsella et al., 1994). None of the control rats produced antibodies against GM1. IgM antibodies from high titer rat sera were also evaluated by TLC-immunostaining for reactivity with GM1 and other gangliosides (Fig. 1) and by Western blotting for reactivity with protein/glycoproteins. As expected, some antibodies were specific for GM1 while others cross-reacted with GD1b that shares Galβ1-3GalNAc moiety with GM1 (Fig. 1, Table 1). None of the rat sera containing anti-GM1 antibodies exhibited antibodies against galactocerebroside in ELISA or against myelin proteins/glycoproteins on Western blots.

The immunized animals were weighed weekly and examined regularly for clinical signs of neuropathy. All animals remained healthy without overt neurological signs of neuropathy during the six months following initial immunization. No weight loss was observed.

All rats were sacrificed 24 weeks post-inoculation and subjected to pathological examination for evidence of demyelination and axonal damage in peripheral nerves. We examined spinal cords, roots, sciatic nerves and dorsal root ganglia. There were no overt signs of pathology in the examined nerves and roots. Fig 2. depicts sciatic nerve sections from a control and GM1 immunized rat and is a representative of all regions examined in which no overt signs of pathology were noted.

4. Discussion

Several previous attempts to induce neuropathy by active immunization of rabbits with GM1 or with *Campylobacter jejuni* lipooligosaccharides (*C. jejuni* LOS) have produced conflicting results. Immunization of New Zealand white rabbits with GM1 developed subclinical neuropathy (Thomas et al., 1991). However, immunization of Japanese white rabbits with GM1 or bovine brain ganglioside mixture induced acute motor axonal neuropathy (Yuki et al., 2001). In two recent studies, however, immunization of New Zealand white rabbits with GM1 induced high titer anti-GM1 antibodies but did not induce peripheral neuropathy (Lopez et al., 2002; Dasgupta et al., 2004). Immunization of rabbits (Ritter et al., 1996; Ang et al., 2000), rats (Wirguin et al., 1997) and mice (Lee et al., 2004) with *C. jejuni* LOS induced high titer anti-GM1 antibodies but not neuropathy. Yuki and associates, however, induced anti-GM1 IgG antibodies and neuropathy in Japanese white rabbits by immunizing them with *C. jejuni* LOS mixed with KLH and emulsified in CFA (Yuki et al., 2004). The reasons for these discrepancies are unknown but may be due to species differences, immunization protocols or antibody affinity (Lopez et al., 2002; Willison and Yuki, 2002; Susuki et al., 2004).

All of our rats immunized with GM1 developed IgM anti-GM1 antibodies. However, the immunized rats did not develop overt signs of neuropathy. Moreover, pathological examination did not reveal any abnormalities in the peripheral nerves. The absence of peripheral nerve damage in rats despite anti-GM1 IgM antibodies could be due to several factors. First, the titers and affinity of anti-GM1 antibodies in the rats may be low compared with antibodies in humans with neuropathy (Lopez et al., 2002). The IgM titers of 1:400 to 1:3,200 in the rats are low compared to anti-GM1 IgM titers in some patients with motor neuropathy (Pestronk et al., 1990; Kinsella et al., 1994; Taylor et al., 1996).

Second, an intact blood nerve-barrier may prevent access of IgM antibody, a much larger molecule than IgG, to its target in the nerve. The importance of blood-nerve barrier in neural injury has been demonstrated by several groups (Pollard et al., 1995; Spies et al., 1995; Hadden et al., 2001; Sheikh et al., 2004). Third, the duration of the experiments may have been relatively short to observe any signs of neuropathy in rats. Some rabbits immunized repeatedly with galactocerebroside did not exhibit clinical signs of neuropathy for more than 10 months after initial inoculation (Saida et al., 1979).

Our results have demonstrated that immunization of Lewis rats with GM1 ganglioside induced only IgM anti-GM1 antibodies despite repeated immunization. Our results are in agreement with previous studies in which only IgM anti-GM1 antibodies were induced in rats (Wirguin et al., 1997) and mice (Freimer et al., 1993). Although our rats did not develop IgG responses to GM1 ganglioside despite repeated immunization, anti-GM1 antibodies in patients with Guillain-Barré syndrome are mainly of IgG1 and IgG3 subclasses, suggesting the role of T cells in antibody response (Ogino et al., 1995; Yuki et al., 1995; Ilyas et al., 2001). While the reason for this discrepancy is unknown, it could be related to species differences in the

presentation of glycolipid antigens to T cells. It is now well established that CD1 molecules present glycolipids to T cells (Porcelli and Modlin, 1999; De Libero and Mori, 2005). CD1 molecules have limited polymorphism and are classified on the basis of their sequence similarity into two groups (Porcelli and Modlin, 1999). In humans, group I consists of CD1a, CD1b and CD1c; group II consists of CD1d only. Mice and rats are unusual among mammals in only expressing group II CD1d molecules (Porcelli and Modlin, 1999). Moreover, in humans, GM1 ganglioside has been shown to be presented to T cells by CD1b (Shamshiev et al., 1999, 2000). CD1 molecules are expressed on dendritic cells (DCs) and on a fraction of B cells in humans and double-negative CD1-restricted T cells are known to help IgG antibody production (Porcelli and Modlin, 1999; Sieling et al., 2000). Therefore, it is tempting to speculate that in humans, GM1 binds to CD1b molecules and activates GM1 reactive, CD1-restricted T cells, which provide support for class switching of anti-GM1 IgM to IgG in B cells, whereas in rats, immunoglobulin class switching of anti-GM1 antibody does not occur because rats do not express CD1b molecules and thus cannot present GM1 to T cells. Interestingly, rabbits also express CD1b molecules (Calabi et al., 1989) and immunization of these species with GM1 can induce anti-GM1 IgG antibodies (Yuki et al., 2001; Lopez et al., 2002).

In conclusion, we have demonstrated that immunization of Lewis rats with GM1 induced only IgM anti-GM1 antibodies despite repeated immunization with GM1 for five months. However, none of the immunized animals exhibited overt clinical signs of neuropathy during six months following initial immunization. Our data suggest that rats are probably not suitable laboratory animals for induction of experimental neuropathy associated with anti-GM1 antibodies.

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Fig. 1. Binding of rat antibodies to gangliosides. In panels A-C, lane 1 contained bovine brain ganglioside standards (10 µg) and lane 2 contained purified GM1 (2 µg). Panel A depicts orcinol stained glycolipids. The other panels were immunostained with 1:100 dilution of rat sera and peroxidase-conjugated goat anti-rat IgM (µ-chain specific). Test sera were from rat #3 (panel B) and rat #14 (panel C).

Fig. 2. One micrometer thick Spurr's resin (epoxy), cut transversely through the sciatic nerve at middle part of thigh segment. (A) Normal sciatic nerve of rat as control. (B) Sciatic nerve of rat immunized with GM1. Both are toluidine blue-stained (x 400).

Table 1
Anti-GM1 IgM antibody titers in Lewis rats immunized with GM1

Rat #	Immunization	Antibody reactivity with glycolipids			
	Antigen	Adjuvant	GM1	GD1b	GalC
R1	GM1/KLH	FA	400	0.0	0.0
R2	GM1/KLH	FA	800	0.0	0.0
R3	GM1/KLH	FA	1,600	800	0.0
R4	GM1/KLH	FA	1,600	0.0	0.0
R5	GM1/KLH	FA	1,600	200	0.0
R6	GM1/KLH	FA	800	0.0	0.0
R7	GM1/KLH	FA	800	0.0	0.0
R8	GM1/KLH	FA	800	0.0	0.0
R9-12	KLH	FA	0.0	0.0	0.0
R13	GM1-liposomes	---	1,600	200	0.0
R14	GM1-liposomes	---	3,200	0.0	0.0
R15	GM1-liposomes	---	1,600	200	0.0
R16-17	Liposomes	---	0.0	0.0	0.0

FA, Freund's adjuvant (First injection in complete FA, all subsequent injections were in incomplete FA); Galc, galactocerebroside; KLH, Keyhole limpet hemocyanin. Antibody titers were determined by an ELISA.