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Induction of Experimental Ataxic Sensory Neuronopathy in Cats by Immunization with Purified SGPG

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Summary

IgM paraproteins in about 50% of the patients with neuropathy associated with IgM gammopathy react with carbohydrate moieties in myelin associated glycoprotein (MAG) and in sulfated glucuronic glycolipids (SGGLs) in human peripheral nerves. However, the role of anti-MAG/SGGL antibodies in the pathogenesis of neuropathy remains unclear. In order to induce an animal model of neuropathy associated with anti-MAG/SGGL antibodies, cats were immunized with sulfoglucuronyl paragloboside (SGPG). *All* four cats immunized with SGPG developed clinical signs of sensory neuronopathy within *11* months after initial immunization, characterized by unsteadiness, falling, hind limb weakness and ataxia. In two cats the ataxia and hind limb paralysis were so severe that the animals had to be euthanized. Pathological examination revealed sensory ganglionitis with inflammatory infiltrates in the dorsal root ganglia. No overt signs of pathology were noted in the examined roots or nerves. High titer anti-SGPG/MAG antibodies were detected in all 4 cats immunized with SGPG but not in 3 control cats. Our data demonstrate that immunization of cats with SGPG induced anti-SGPG antibodies and sensory neuronopathy clinically resembling the sensory ataxia of patients with monoclonal IgM anti-MAG/SGPG antibodies. This study suggests that these anti-MAG/SGPG antibodies play a role in the pathogenesis of this neuropathy.

1. Introduction

Paraproteinemic neuropathies are a diverse group of disorders in which there is an excessive amount of monoclonal antibody termed a paraprotein, and are also called monoclonal gammopathies. IgM is the most common paraprotein in patients with neuropathy and in more than seventy percent of these patients IgM paraproteins react with oligosaccharide moieties of glycoproteins and glycolipids (Latov 1995; Quarles and Weiss, 1999, Quarles 2007; Willison and Yuki, 2002; Nobile-Orazio, 2004; Ilyas, In press). IgM paraproteins in about 50% of the patients with neuropathy associated with IgM gammopathy react with carbohydrate moieties in several myelin glycoproteins including myelin-associated glycoprotein (MAG), P0 glycoprotein and peripheral myelin protein-22 (PMP-22) and also in sulfated glucuronic glycolipids (SGGLs) in human peripheral nerves (Ilyas et al., 1984, Ilyas, In press; Latov 1995; Quarles and Weiss, 1999, Quarles, 2007; Willison and Yuki, 2002; Nobile-Orazio, 2004). These glycoconjugates also react with a mouse monoclonal antibody HNK-1 (Leu-7) directed against human natural killer cells (Chou et al., 1986; McGarry et al., 1983). Two SGGLs from human peripheral nerves have been characterized. They are sulfated glucuronyl

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paragloboside (SGPG) and sulfated glucuronyl lactosaminyl paragloboside (SGLPG) (Chou et al., 1986; Ariga et al., 1987). The terminal sulfated glucuronic acid in SGPG is a critical part of the epitope for all anti-MAG/SGPG IgM paraproteins and for monoclonal HNK-1 antibody (Ilyas et al., 1986, 1990, 1992).

Most patients with anti-MAG/SGPG IgM paraproteins have a chronic, slowly progressive, predominantly sensory, ataxic, demyelinating neuropathy (Nobile-Orazi et al., 1994; Van den Berg et al., 1996; Chassande et al., 1998). Neurophysiological examination typically shows a distal accentuation of conduction slowing (Kaku et al., 1994). Sural nerve biopsies have revealed segmental demyelination without inflammatory infiltrates, and deposits of IgM and complement on myelinated fibers. Myelin widening is the hallmark of neuropathy associated with anti-MAG IgM paraproteinemia (Mendell et al., 1985; Vital et al., 1989).

Despite a large body of evidence that suggests anti-MAG antibodies are pathogenic, the precise role of anti-MAG/SGPG antibodies in the pathogenesis of neuropathy remains unknown. Attempts to produce experimental models of neuropathy by active immunization of rabbits and rats with SGPG have not been successful (Kohriyama et al., 1988; Maeda et al., 1991b; Yamawaki et al., 1996; Ilyas et al., 2002), presumably due to the fact that rabbits and rats express low levels of SGGLs as compared with humans (Ilyas et al., 1986). Unlike rabbits and rodents, cats express high levels of HNK-1 epitope on myelin proteins (O'Shannessy et al., 1985) and high levels of SGGLs in peripheral nerves (Ilyas et al., 1986). The objective of the current study was to induce an experimental model of neuropathy by immunizing cats with purified SGPG. Portions of this work have been presented in abstract form (Ilyas et al., 2007).

2. Materials and Methods

2.1. Reagents

Bovine brain ganglioside mixture, GM1, GD1b, galactocerebroside and sulfatide 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. Freund's adjuvant was obtained from Difco Laboratories, Detroit, MI. Bovine cauda equina was purchased from Pel Freez Biologicals, Rogers, AZ.

2.2. Antibodies

Mouse anti-MAG monoclonal antibody, B11F7, was produced as described previously (Doberson et al., 1985). This antibody reacts with the protein part of MAG. HRP-conjugated goat anti-cat IgM (μ -chain specific) or IgG (γ -chain specific) were purchased from (Bethyl Laboratories Inc., Montgomery, TX).

2.3. Animals

Female domestic shorthaired crossbred cats (4-5 months) were purchased from Liberty Laboratories, Waverly, NY. All animal research experiments were performed according to the institutional guidelines on animal welfare and were approved by the Institutional Animal Care and Use Committee (IACUC) at New Jersey Medical School.

2.4. Purification of SGPG

SGPG was isolated and purified from bovine cauda equina as described previously (Ilyas et al. 2002). The purity of SGPG was checked by thin-layer chromatography and orcinol spray.

2.5. Animal Immunization

Four cats (*C-1 - C-4*) (Table 1) were injected subcutaneously at multiple sites in the back (thoracic region) with 200 µg of purified SGPG mixed with keyhole limpet hemocyanin (KLH) (2 mg/ml) and emulsified with equal volume of complete Freund's adjuvant (CFA). The cats were given booster injections every 3 weeks with 100 µg SGPG mixed with KLH in incomplete Freund's adjuvant (IFA). Three control cats (*C-5-C-7*) (Table 1) were injected with KLH mixed with CFA only and then were given booster injections every 3 weeks with KLH emulsified with IFA. The animals were examined regularly for clinical symptoms and weighed weekly. Animals were bled prior to initial immunization and then every 3 weeks thereafter.

2.6. Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed essentially as described (Ilyas and Chen, 2007) except that the antigens were SGPG and sulfatide and primary antibodies used were cat sera and the secondary antibodies were peroxidase-conjugated goat anti-cat IgM and IgG antibodies. Briefly, 100 nanograms of purified SGPG or sulfatide 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 2 h. The wells were emptied and 100 µl of cat serum diluted 1:100 in PBS containing 1% BSA 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-cat 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.

2.7. Thin-layer chromatogram (TLC)-immunostaining

Bovine brain gangliosides and SGGLs 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 2 N H₂SO₄). The binding of anti-SGPG 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 30 s and then dried. The plates were then overlaid with 1% BSA in PBS for 2 h in the cold and then with cat serum (1:100 dilution) for 4 h at 4°C. The plates were washed with cold PBS and then overlaid with peroxidase-conjugated anti-cat µ or γ-chain specific antibodies and kept at 4°C overnight. The plates were then washed and developed with o-phenylenediamine.

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

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., 1992) except that bovine brain myelin proteins were used as antigens and the blots were incubated with cat sera followed by peroxidase-conjugated goat anti-cat IgG or IgM antibody or with mouse monoclonal IgG antibody followed by peroxidase-conjugated goat anti-mouse IgG. The immunoblots were developed by using the ECL system (Pierce, Rockford, IL).

2.9. Histological examination

The animals were anesthetized with sodium pentobarbital (50 mg/kg) intraperitoneally and perfused through the left cardiac ventricle with 4% paraformaldehyde in 0.1M phosphate buffered saline. Sciatic nerves, and dorsal ganglia were removed from both sides. The spinal cord with roots was dissected. The tissues were immediately immersed in 4% paraformaldehyde solution and subsequently embedded in paraffin. Sections were analyzed for histological examination using hematoxylin & eosin, Bielschowsky stain and Luxol fast blue.

3. Results

3.1. Induction of IgM and IgG anti-SGPG/MAG antibodies

All four cats immunized with purified SGPG exhibited strong antibody response to both SGPG and its higher homologue, SGLPG (Fig. 1 and 2). The serum anti-titers in cat C-1 were 12,800 for IgM and 6,400 for IgG. Antibody titers in cat C-2 were 6,400 for IgM and 3,200 for IgG. Antibody titers in cat C-3 were 25,600 for IgM and 6,400 for IgG. In cat C-4, the highest antibody titers were 25,600 for IgM and 6,400 for IgG. Antibody titers in this cat, however, fell to 3,200 for IgG (Fig 1). Although anti-MAG/SGPG antibodies in our cats are relatively high, they are lower than anti-SGPG IgM titers of more than 64,000 in patients with neuropathy associated with gammopathy. Anti-SGPG antibodies were not detected in 3 control cats immunized with KLH only. As expected, TLC-immunostaining showed that antibodies from all four SGPG-immunized cats cross-reacted with SGLPG, a higher homologue of SGPG but did not cross-react with gangliosides in bovine brain ganglioside mixture on TLC (Fig. 2). The cat antibodies also did not cross-react with galactocerebroside and sulfatide in ELISA (data not shown). Cat sera were also tested by Western blotting for reactivity with protein/glycoprotein antigens (Fig. 3). Anti-SGPG antibodies from all four cats immunized with SGPG cross-reacted with MAG in bovine brain myelin (Fig. 3). Thus, the cat serum anti-SGPG antibodies resembled human IgM paraproteins from patients with neuropathy by reacting with SGPG, SGLPG and bovine MAG.

3.2. SGPG immunized cats develop clinical signs of neuropathy

Four of 4 cats immunized with SGPG developed clinical signs of neuropathy within *11* months after initial immunization (Table 1). One cat, C-1, began to exhibit mild hind legs paresis beginning at 68 days after initial immunization, and rapidly progressed to severe ataxia, falling, full hind limb paralysis (Fig. 4A), moderate front limb weakness, and rapid breathing. This cat exhibited a tendency to hide. In this cat the ataxia and hind limb paralysis were so severe that the animal had to be euthanized. Cat C-2 exhibited clinical symptoms of neuropathy at 163 days after initial immunization. In this cat, neuropathy also developed rapidly and was characterized by ataxia, unsteadiness and hind leg paralysis. This cat was lethargic, favored right front limb when she moved, and when sitting, seemed to lean towards the left. The cat developed respiratory distress and was euthanized. Cat C-3 exhibited clinical symptoms 239 days after initial immunization and like cat C-1, rapidly progressed to severe ataxia, falling, hind limb paralysis and front limb weakness (Fig. 4B-D). *Cat C-4, exhibited mild front limb weakness 329 days after initial immunization. A week later, neuropathy rapid progressed to severe ataxia and paralysis of both front and hind limbs.* None of the control cats immunized with KLH only developed neuropathy.

3.3. Pathological examination reveals sensory ganglionitis with inflammatory infiltrates in the dorsal root ganglia

Spinal cords, sciatic nerves, dorsal roots, and dorsal root ganglia of SGPG-immunized and control cats were examined histologically for evidence of inflammation and demyelination.

Pathological examination revealed sensory ganglionitis with inflammatory infiltrates in the dorsal root ganglia of SGPG-immunized animals. Figs. 4E-H, are representative of dorsal root/ganglion depicting lymphocyte infiltrates around the ganglionic neurons and around myelinated fibers. However, there was no inflammation and/or demyelination in the examined spinal cords, roots or sciatic nerves.

4. Discussion

The pathogenic role of anti-MAG/SGPG antibodies in peripheral neuropathy has been investigated by passive immunization of animals. Injection of serum from paraproteinemic patients with neuropathy and anti-MAG/SGPG IgM paraproteins and complement into feline and rabbit peripheral nerve produced myelin destruction (Hays et al., 1987; Willison et al., 1988; Monaco et al., 1995). Systemic transfusion of anti-MAG paraproteins was shown to cause segmental demyelination in the chicken (Tatum, 1983). Intraneural injections of rat anti-SGPG antibodies induced demyelination in rat sciatic nerve, along with mild to moderate clinical symptoms (Maeda et al., 1991a).

The pathogenic role of anti-MAG/SGPG antibodies in peripheral neuropathy has also been investigated by active immunization of animals with purified MAG and SGPG. Immunization of rabbits with purified SGPG produced mild neurological symptoms and electrophysiological dysfunction (Kohriyama et al., 1988), but demyelination of peripheral nerves was not observed. Immunization of Lewis rats with SGPG induced high titer anti-SGPG antibodies (Maeda et al., 1991b; Yamawaki et al., 1996), but there were no overt signs of nerve damage. However, nerve conduction abnormalities were observed in rats in one study (Yamawaki et al., 1996), but this report conflicts with another report from the same laboratory in which nerve dysfunction was not detected (Maeda et al., 1991b). In another study, immunization of Lewis rats with purified SGPG produced high levels of anti-SGPG antibodies but did not produce any clinical or pathological changes in the immunized animals (Ilyas et al., 2002). One reason for the failure to induce experimental neuropathy in the rats and rabbits is that these animals express very low levels of the HNK-1 epitopes on glycoproteins in the peripheral nervous system and express very low levels of SGGLs (O'Shannessy et al., 1985; Ilyas et al., 1986).

In a previous study, immunization of cats with human MAG did not induce peripheral neuropathy apparently due to the fact that the immunized cats developed antibodies to the peptide part of MAG but not to the crucial HNK-1 oligosaccharide moiety that is recognized by anti-MAG/SGPG antibodies in patients with neuropathy (Kahn et al., 1989). However, immunization of cats with purified cat MAG did induce low levels of IgM and IgG antibodies that reacted with SGPG and carbohydrate epitopes in MAG, but did not cause neuropathy in the animals (Willison, Ilyas and Quarles, 1987, unpublished results). In our current study, all four cats immunized with SGPG produced high titer anti-SGPG antibodies demonstrating that SGPG is a better immunogen than human MAG for generating a relatively strong immune response in cats. Induced antibodies from all four SGPG immunized cats reacted with the oligosaccharide moieties on SGPG, SGLPG and bovine MAG and thus resembled human IgM paraproteins.

All of our cats immunized with SGPG developed neuronopathy and ataxia, clinically resembling the sensory ataxia of patients with monoclonal IgM anti-MAG/SGPG antibodies (Nobile-Orazio et al., 1994). Sensory ataxic neuropathy is the clinical hallmark in several patients with IgM paraprotein reacting with gangliosides containing disialosyl moieties (Ilyas et al., 1985; Dalakas and Quarles, 1996; Willison et al., 2001). Pathological examination of dorsal root ganglia of our SGPG-immunized cats revealed sensory ganglionitis with inflammatory infiltrates. Histology of dorsal root ganglia from neuropathy patients with anti-MAG/SGPG IgM paraproteins essentially has not been examined. However, inflammation in

paraneoplastic subacute sensory neuronopathy has been demonstrated (Wanschitz et al., 1997). Since SGPG and SGLPG are enriched in human dorsal root ganglia (Ariga et al., 1990), and blood-nerve barrier is permeable at the level of the dorsal root ganglia (Olsson, 1968; Abrams et al., 2006), the anti-MAG/SGPG antibodies may enter the dorsal root ganglia and cause nerve dysfunction. The reason for the lack of overt signs of demyelination or axonal damage in peripheral nerves of cats is unknown but could be due to several factors. First, our immunization protocol might not generate adequate cellular immune response that opens the blood-nerve barrier to antibodies (Pollard et al., 1995; Hadden et al., 2002) and allows access of anti-MAG/SGPG antibodies throughout the peripheral nerve to their targets. Second, demyelination and axonal damage may require more time to develop with chronic and higher levels of anti-MAG/SGPG antibodies (Meucci et al., 1999). The present experimental data, however, provide support for the possibility that anti-MAG/SGPG neuropathy may be a primary neuronopathy with secondary nerve degeneration.

In conclusion, immunization of cats with SGPG induced anti-SGPG antibodies and sensory neuronopathy clinically resembling the sensory ataxia of patients with monoclonal IgM anti-MAG/SGPG antibodies. *This study suggests that these anti-MAG/SGPG antibodies play a role in the pathogenesis of this neuropathy.* Further characterization of this feline model could help us understand the disease mechanism and lead to more specific therapeutic strategies.

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Figure 1.
Antibody titers in sera of cats immunized with SGPG

Figure 2. Binding of cat antibodies to sulfoglucuronyl glycolipids. Lane 1 in each panel contained bovine brain gangliosides for reference. Lane 2 in each panel contained purified SGPG and SGLPG. Panel A depicts orcinol stained glycolipids. Panels B and C depict TLC after overlay with serum from a cat immunized with SGPG (C-1) followed by peroxidase-labeled goat anti-cat IgM (μ chain specific) and peroxidase-labeled goat anti-cat IgG (γ chain specific), respectively.

Figure 3. Immunoblots showing binding of cat antibodies to bovine MAG. Western blot following 12 % SDS-PAGE of bovine CNS myelin proteins (80 μ g / lane). (Lane 1) stained with a 1:500 dilution of serum from cat C-1, followed by HRP-conjugated goat anti-cat IgM. (Lane 2) stained with a 1:500 dilution of a mouse monoclonal antibody (B11F7) followed by HRP-conjugated goat anti-mouse IgG.

Figure 4. SGPG immunized cats with neuropathy. (A) Cat C-1 had severe ataxia and hind limb weakness. (B,C,D) Cat C-3 had hind limb weakness, severe ataxia and difficulty walking. She was slipping and falling. (E, F) Hematoxylin & eosin stained cross section of root/dorsal root ganglion of cat C-2 demonstrates lymphocytic infiltrates around the ganglionic neurons. (G, H) Bielschowsky stain: Dorsal root/ganglia, corresponding to (E,F) shows inflammation around myelinated fibers.

Table 1

Cats immunized with SGPG and with Adjuvant only

Cat	Immunogen	Number of injections [¶]	Neurological symptoms	Disease onset [#]	Anti-SGPG IgM/ IgG titer
C-1	SGPG/KLH/FA	4	+	68	12,800/6,400
C-2	SGPG/KLH/FA	8	+	163	6,400/3,200
C-3	SGPG/KLH/FA	12	+	239	25,600/6,400
C-4	SGPG/KLH/FA	16	+	329	25,600/3,200
C-5	KLH/FA	8	-	-	0/0
C-6	KLH/FA	12	-	-	0/0
C-7	KLH/FA	12	-	-	0/0

SGPG, sulfated glucuronyl paragloboside; KLH, keyhole limpet hemocyanin; FA, Freund's adjuvant.

Antibody titers are at the time of appearance of clinical symptoms. Anti-SGPG antibodies were not detected in control cats (C-5, C-6, and C-7).

[¶]First injection was in complete FA, all subsequent injections were in incomplete FA

+ animals exhibited neurological symptoms

- no apparent neurological symptoms

[#] disease onset days after initial immunization.