Localization of major gangliosides in the PNS: implications for immune neuropathies

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Summary
Antibodies targeting major gangliosides that are broadly distributed in the nervous system are sometimes associated with clinical symptoms that imply selective nerve damage. For example, anti-GD1a antibodies are associated with acute motor axonal neuropathy (AMAN), a form of Guillain–Barre syndrome that selectively affects motor nerves, despite reports that GD1a is present in human axons and myelin and is not expressed differentially in motor versus sensory roots. We used a series of high-affinity monoclonal antibodies (mAbs) against the major nervous system gangliosides GM1, GD1a, GD1b and GT1b to test whether any of them bind motor or sensory fibres differentially in rodent and human peripheral nerves. The following observations were made. (i) Some of the anti-GD1a antibodies preferentially stained motor fibres, supporting the association of human anti-GD1a antibodies with predominant motor neuropathies such as AMAN. (ii) A GD1b antibody preferentially stained the large dorsal root ganglion (DRG) neurones, in keeping with the proposed role of human anti-GD1b antibodies in sensory ataxic neuropathies. (iii) Two mAbs with broad structural cross-reactivity bound to both gangliosides and peripheral nerve proteins. (iv) Myelin was poorly stained; all clones stained axons nearly exclusively. Our findings suggest that anti-ganglioside antibody fine specificity as well as differences in ganglioside accessibility in axons and myelin influence the selectivity of injury to different fibre systems and cell types in human autoimmune neuropathies.

Keywords: Guillain–Barre syndrome; anti-ganglioside antibodies; AMAN; sensory ataxic neuropathy; immune neuropathies

Abbreviations: ABC = avidin–biotin–horseradish peroxidase complex; ABC-AP = avidin–biotin–alkaline phosphatase; AMAN = acute motor axonal neuropathy; DRG = dorsal root ganglion; ELISA = enzyme-linked immunosorbent assay; FITC = fluorescein isothiocyanate; HRP = horseradish peroxidase; IgG = immunoglobulin G; IgM = immunoglobulin M; mAb = monoclonal antibody; TLC = thin-layer chromatography

Introduction
Gangliosides, sialic acid-containing glycosphingolipids highly enriched in the vertebrate nervous system (Yu and Saito, 1989), are implicated as target antigens in various autoimmune neurological disorders (Hartung et al., 1996; Yuki, 1998; Hughes et al., 1999; Quarles and Weiss, 1999; Kusunoki, 2000; O’Leary and Willison, 2000; Yuki, 2000; Ariga et al., 2001). Different lines of investigation have found strong evidence for the pathogenic role of anti-ganglioside antibodies in several immune neuropathies, particularly in some forms of Guillain–Barre syndrome (Roberts et al., 1994, 1995; Kusunoki et al., 1996; Buchwald et al., 1998; Goodyear et al., 1999; Paparounas et al., 1999; Plomp et al., 1999; Yuki et al., 2001). The anti-ganglioside antibodies in patients with Guillain–Barre syndrome are usually high-affinity complement-fixing immunoglobulin G (IgG) (Ogino et al., 1994; Willison and Veitch, 1994; Yuki et al., 1995). Recently, clear correlations between specific anti-ganglioside antibodies and different variants of Guillain–Barre syndrome have emerged. For example, antibodies to GD1a, GalNAc-GD1a and GM1 are associated with acute motor axonal
neuropathy (AMAN) or motor variants of Guillain–Barré syndrome (Ilyas et al., 1988a; Yuki et al., 1990, 1993a, 1996, 1999; Ho et al., 1995; Lugaresi et al., 1997; Kuwabara et al., 1998; Ho et al., 1999; Kaida et al., 2000; Ogawara et al., 2000) and antibodies to GQ1b are associated with Fisher syndrome (Chiba et al., 1992, 1993; Willison et al., 1993; Yuki et al., 1993b). In contrast to motor neuropathies, some acute and chronic sensory ataxic neuropathies are associated with antibodies to GD1b, including those that cross-react with other disialosyl gangliosides (Willison et al., 1994; O’Leary and Willison, 1997; Eurelings et al., 2001; Pan et al., 2001; Susuki et al., 2001).

One frequently noted enigma is the observation that antibodies recognizing ubiquitous gangliosides are sometimes associated with clinical symptoms that imply specific cellular injury, such as motor axons in AMAN. Gangliosides are present in both axons and myelin, and there are no significant quantitative differences between the major gangliosides in human ventral and dorsal roots (Svennerholm et al., 1992, 1994; Ogawa-Goto and Abe, 1998) that might explain the different ganglioside associations with primary axonal injury or predominant motor or sensory fibre damage. Moreover, some anti-GM1 antibodies recognize the Gal β3 GalNAc moiety shared by GM1, GD1b (Walsh et al., 1991) and some peripheral nerve glycoproteins (Apostolski et al., 1994). This finding has raised the question whether anti-ganglioside antibodies recognize gangliosides alone or cross-react with other glycoconjugates in peripheral nerves.

Immunolocalization studies with patient sera or with affinity-purified patient antibodies would help experimentally address the issue of the selective nerve fibre injury associated with anti-ganglioside antibodies. However, patients’ sera cannot be used reliably in localization studies because antibodies in normal sera bind to peripheral nerves (Stefansson et al., 1985; Paparounas et al., 1999; K. Sheikh, unpublished observations), and affinity purification techniques for anti-glycolipid antibodies are inefficient. In the absence of experimentally useful human anti-ganglioside antibodies, high-affinity IgG-class mouse anti-ganglioside monoclonal antibodies (mAbs) with well-defined specificities similar to those found in Guillain–Barré syndrome would be useful in probing the pathogenetic sequence of this disease. Although prior attempts to raise IgG-class mAbs against the major brain gangliosides in mice were problematic, we found recently that mice genetically engineered to lack complex gangliosides were excellent hosts for raising anti-ganglioside antibodies (Lunn et al., 2000). Subsequently, we generated and characterized a family of IgG-class mouse mAbs against the four major human brain gangliosides: GM1, GD1a, GD1b and GT1b (Schnaar et al., 2002). Mice genetically engineered to specifically lack complex gangliosides were also used to test whether anti-ganglioside antibodies exclusively recognize gangliosides or also cross-react with glycoproteins in peripheral nerves (see Results).

The present study describes the distribution of the four major nervous system gangliosides (GM1, GD1a, GD1b and GT1b) in the PNS, as determined by newly generated high-affinity IgG anti-ganglioside antibodies (Schnaar et al., 2002). Gangliosides GM1, GD1a and GD1b were selected because of their association with different variants of Guillain–Barré syndrome. Immunolocalization of gangliosides was compared with that of biochemically extracted glycolipids from peripheral nerves. We report that certain anti-ganglioside antibodies with well-defined specificities bind selectively to distinct fibre systems and cell types in the PNS. These findings support the hypothesis that anti-ganglioside antibodies have the potential to cause preferential injury to distinct neuronal and nerve fibre populations.

**Material and methods**

**Reagents and tools**

**Gangliosides**

Purified gangliosides GM3, GD3, GM2, GA1 (asialo-GM1), GM1, GD1a, GD1b, GT1b and GQ1b were from Matreya (Pleasant Gap, PA, USA) or Sigma Chemical (St Louis, MO, USA); synthetic cisGM1 (GM1b), GM1α, GD1α, GT1αα and GQ1bα were kindly provided by Dr Makoto Kiso, Gifu University (Ikami et al., 2000). GalNAc-GD1a was synthesized from GD1a by an enzymatic reaction with a recombinant UDP-GalNAc:GM3/GD3 N-acetylgalactosaminyltransferase, which was the gift of Dr Shawn DeFrees, Neose Technologies, Horsham, PA, USA. The 15 ganglioside
structures are shown in Figure 1; nomenclature is based on that of Svennerholm (Svennerholm, 1994).

**Antibodies and reagents**

Rabbit anti-neurofilament 200 kDa was used for labelling axons and neurones, and biotin-labelled isoelectin B-4 (IB-4) (Sigma) was used to label small non-peptidergic neurones (Silverman and Kruger, 1990) and unmymelinated axons or Remak bundles (Kitchener et al., 1994; Haberberger and Bodenbenner, 2000). Fluoroisothiocyanate (FITC)- or Texas red-conjugated specific anti-mouse IgG and IgM and anti-rabbit IgG (H+L) (Vector Laboratories, Burlingame, CA, USA), biotinylated anti-mouse IgG or IgM (Jackson Immunoresearch), and avidin–biotin–horseradish peroxidase complex (ABC) or avidin–biotin–alkaline phosphatase (ABC-AP) (Vector Laboratories, West Grove, PA, USA), CY5- or Texas red-conjugated streptavidin (Jackson Immunoresearch), and avidin–biotin–horseradish peroxidase (HRP)-conjugated cholera toxin subunit B was from List Biologicals, Campbell, CA, USA.

**Mice lacking complex gangliosides**

Mice were engineered to carry a deletion in the gene coding the ganglioside-specific glycosyltransferase UDP-GalNAc:GM3/GD3 β-4-N-acetylgalactosaminyltransferase (GalNAcT), as described previously (Liu et al., 1999). The resulting gene-deleted mice (GalNAcT−−) failed to express any of the major brain gangliosides—GM1, GD1a, GD1b, or GT1b—and instead expressed GD3 and GM3. These animals were used to generate anti-ganglioside monoclonal antibodies and served as negative controls for complex ganglioside staining in tissues. Since the founder strain was repeatedly backcrossed to C57Bl/6 strain mice, the latter were used as wild-type controls.

**Anti-ganglioside antibodies**

The one IgM (see below) and 10 IgG mAbs against the four major gangliosides (GM1, GD1a, GD1b and GT1b) used are designated according to their ganglioside specificity and IgG isotype (1, 2a or 2b); for example, GD1a-1 refers to a mAb with GD1a specificity and IgG1 isotype. The generation and specificity of IgG mAbs were reported in two previous publications (Lunn et al., 2000; Schnaar et al., 2002). In the present study the reactivity of four GD1a-related mAbs to GalNAc-GD1a was also determined by quantitative enzyme-linked immunosorbent assay (ELISA) according to methods described previously (Collins et al., 1997). Furthermore, a previously unreported anti-GM1 IgM mAb was generated and characterized, using methods reported previously, to generate anti-GM1 IgG mAbs (Schnaar et al., 2002), except that hybridomas were screened with an anti-mouse IgM secondary antibody.

**Antibody purification**

Hybridomas were grown to high density in Dulbecco’s modified Eagle medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10 mM HEPES, 200 U/l insulin, 3.5 μl/l β-mercaptoethanol, 150 mg/l oxaloacetic acid, 50 mg/l sodium pyruvate, 2 mM glutamine and 10% (by volume) fetal bovine serum (Hyclone, Logan, UT, USA). Secreted IgG mAbs were purified from cell-culture supernatants by affinity chromatography using immobilized goat anti-mouse IgG agarose (Sigma). All procedures were performed at 4°C. Supernatant (50 ml) was loaded onto 2.5 ml of affinity resin, the resin was washed with 10 mM sodium phosphate, 0.5 M sodium chloride, pH 7.2, and mAb was eluted with 100 mM glycine, 150 mM sodium chloride, pH 2.4. Eluate fractions were immediately neutralized by addition of one-tenth volume of 1 M sodium phosphate buffer, pH 8.6. Protein-containing fractions, identified by their UV absorbance at 280 nm, were pooled and dialysed against calcium- and magnesium-free Dulbecco’s PBS (phosphate-buffered saline). Samples were concentrated to >1 mg/ml protein by Centricon ultrafiltration (Millipore, Bedford, MA, USA), sterilized by filtration and stored at −20°C until used.

**Ganglioside extraction and TLC immuno-overlay assays**

Extraction of gangliosides from human dorsal and ventral roots was as described previously (Schnaar, 1994). Human tissues from four autopsies collected within 24–48 h post-mortem and dissected into dorsal and ventral roots were homogenized with a Polytron in 10 ml of ice-cold water. Methanol then chloroform were added to give chloroform–methanol–water (4 : 8 : 3), and the tissues were extracted by stirring the mixture at ambient temperature. Insoluble material was removed by centrifugation, and water was then added to the supernatant to give chloroform–methanol–water (4 : 8 : 5.6). The resulting phases were separated by centrifugation and the upper phase, containing gangliosides, was desalted using a Sep-Pak C18 cartridge. Finally, phospholipids were removed by partitioning in diisopropyl ether–butanol–50 mM aqueous NaCl (6 : 4 : 3) as described (Ladisch and Gillard, 1985), and desalting was repeated. Gangliosides were also extracted from pooled spinal roots and peripheral nerves from 12 wild-type and five GalNAcT−− adult mice. Only the sciatic nerves from wild-type animals were pooled, but both the sciatic and brachial plexus nerves of the transgenic mice were used because of their limited number. The final partition was not performed on rodent gangliosides because of the limited quantity.

Extracted gangliosides from all species were analysed by thin-layer chromatography (TLC) (HPTLC Silica Gel 60; Merck, Darmstadt, Germany) with chloroform–methanol–0.25% aqueous KCl (60 : 35 : 8) as running solvent. Gangliosides were detected with a resorcinol–HCl–Cu2+ reagent (Schnaar and Needham, 1994). Bovine brain
gangliosides GM1, GD1a, GD1b and GT1b were used as standards. TLC plates were scanned and gangliosides were quantified as described previously (Schnaar and Needham, 1994).

TLC immuno-overlay studies were done on ventral and dorsal root gangliosides extracted from human samples and on peripheral nerve gangliosides extracted from mice. Ganglioside extracts from human dorsal and ventral roots and mouse peripheral nerves (~500–600 pmol/lane) were resolved by TLC as described above. The dried plates were immersed in 0.2% polyisobutylmethacrylate in hexane for 30 s and air-dried. The coated plates were sprayed with PBS until wet and then immersed in PBS containing 1% bovine serum albumin (blocking buffer) for 1 h. Purified mAbs (0.3–3.5 μg/ml) were diluted in blocking buffer and transferred to the surface of the TLC plates. After incubation for 2 h at ambient temperature in a humidified chamber, the plates were washed three times with PBS, secondary antibody (alkaline phosphatase-conjugated goat anti-mouse IgG or IgM 0.6 μg/ml in blocking buffer) was applied to the TLC plates, and the plates were incubated for 1 h. After incubation with secondary antibody, the plates were washed three times with PBS and once with developing buffer (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl2). Finally, plates were immersed in a nitroblue tetrazolium/bromochloroindolyl phosphate (1 and 0.5 mg/ml, respectively, in developing buffer) solution. Upon the appearance of purple bands, plates were washed in water and dried. In separate studies, extracted human ventral and dorsal root gangliosides were resolved on TLC plates and then treated with Vibrio cholerae neuraminidase (10 mU/ml, for 2 h at 37°C), which converts GD1a, GD1b and GT1b to GM1. These TLC plates were immunostained with mAb GM1-1 (2.3 μg/ml).

**Immunocytochemistry**

Rat tissues were optimal for detailed immunohistochemical analyses. Lack of optimal tissue preservation and autofluorescence were problematic with human tissues because of the post-mortem interval. Background secondary antibody binding was greater in the mouse tissue, due to endogenous mouse IgGs. Therefore, studies on human and mouse tissues were restricted. Purified anti-ganglioside mAbs (10–20 μg/ml) were used for most of the immunohistochemical studies. When purified antibodies were not available, hybridoma supernatants with high titres, as determined by ELISA, were used. Hybridoma supernatants were used exclusively for all studies with GM1-IgM and GT1b-2a. Preliminary studies established that spinal root and peripheral nerve staining was reduced significantly after fixation with paraformaldehyde, but spinal cord staining was affected only minimally. Therefore, fresh frozen tissue was used for all PNS immunocytochemistry, including peripheral nerves, spinal roots and dorsal root ganglia, except for teased fibres. Fixed tissues, however, were generally used for spinal cord staining.

**Tissue collection and preparation**

Cauda equina, lumbar dorsal root ganglia (DRGs), lumbar spinal cord and sciatic nerves were collected from adult 8- to 12-week-old Sprague–Dawley rats, wild-type mice and GalNAcT+/− transgenic mice. All animals were anaesthetized with Nembutal (60 mg/kg). The animals were perfused through the heart with PBS followed by 10% sucrose for collection of spinal roots, DRGs and sciatic nerves. These tissues were snap-frozen in isopentane at −70°C and cryosectioned. A separate set of animals was perfusion-fixed with 4% paraformaldehyde; lumbar spinal cords were collected and further immersion-fixed overnight. Human lumbar spinal cord, roots and DRGs were obtained from autopsies. Human lumbar spinal cord was immersion-fixed in 4% paraformaldehyde for 6–8 h. Fixed tissues were cryoprotected with serial sucrose solutions (10–30%), snap-frozen and cryosectioned. The 8–10 μm cross-sections and longitudinal sections were collected and air-dried on Superfrost Plus Slides (Electron Microscopy Science, Fort Washington, PA, USA) for immunohistochemical studies. Spinal cord immunostaining was also done on 40 μm, free-floating sections with selected antibodies to compare the staining patterns with those on the 8–10 μm sections.

**Teased fibres**

Rat peripheral nerves were prepared according to methods described previously (Sheikh et al., 1999). Animals were perfused with 2% paraformaldehyde and the spinal roots and sciatic nerves were removed and fixed for an additional 30 min on ice. Nerves were washed in PBS and incubated in 1 mg/ml collagenase type IV in PBS (Sigma) for 30–60 min at ambient temperature. After multiple washes in ice-cold PBS, the nerves were desheathed and teased into small bundles of fibres. These teased fibre preparations were immunostained with individual anti-ganglioside mAbs (10–20 μg/ml) at 4°C overnight and developed with specific secondary antibodies conjugated to FITC. Cholera toxin subunit B conjugated to FITC, which binds preferentially to ganglioside GM1, was used for comparison. The samples were further teased, mounted in glycerol and overlaid with coverslips for confocal microscopy.

**Single labelling**

All tissues from all species were immunostained by the single-labelling technique, according to methods described previously (Sheikh et al., 1999). Briefly, 8–10 μm cryostat cross-sections were quenched for endogenous peroxidases, blocked for non-specific binding and incubated with individual anti-ganglioside mAbs (10–20 μg/ml) at 4°C overnight. The sections were then incubated sequentially with specific biotinylated secondary antibodies 1:100 for 1 h at ambient temperature and with ABC or ABC-AP for 30 min at ambient temperature. Finally, the sections were developed by
measurement. The DRG neurones were classified as small measuring cells more than once, each cell was marked after nucleus was clearly identified. To prevent counting and antibody were measured for diameter at the level where the DRG neurones labelled positively with anti-neurofilament antibodies. The tissue mAb were analysed. Spinal cords were double-labelled with individual anti-ganglioside antibodies (GM1-2b, GD1a-1, GD1a-2b, GD1b-1, GT1b-2b) and more than 100 DRG neurones per section by 100. This measurement was done for six represen-
tative mAbs (GM1-2b, GD1a-1, GD1a-2b, GD1b-1, GT1b-2b and GD1a/GT1b-2b), and more than 100 DRG neurones per mAb were analysed. Spinal cords were double-labelled with anti-ganglioside and anti-neurofilament antibodies. The tissue sections were mounted in anti-fade mount (Biomed, Foster City, CA, USA). Confocal microscopy was performed with an LSM510 confocal microscope system (Carl Zeiss, Jena, Germany) and images were captured using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

Control studies

For negative controls, primary antibody was omitted. For multiple-labelling experiments, control studies were done in a chequerboard pattern to ensure that secondary antibodies/ reagents did not cross-react with each other or with unpaired primary antibodies. Tissue sections were also stained with selected antibodies (GD1a-1, GD1b-1 and GM1-1) after V. cholerae neuraminidase treatment (20 mU/ml for 24 h at 37°C) to determine whether anti-ganglioside antibodies recognized sialic acid-containing glycoconjugates in tissue sections.

Solvent extraction

Cryosections were treated with chloroform–methanol (1 : 1) for 30–60 min at ambient temperature. Solvent-treated and untreated sections were stained with mAbs. Disappearance of antibody staining after solvent treatment was interpreted as evidence that the antigens recognized in tissue sections were glycolipids. Binding with HRP-conjugated cholera toxin subunit B (2–10 μg/ml at 4°C overnight) was used as a positive control for solvent extraction of GM1. Solvent extraction studies were done systematically on rat peripheral nerves and spinal cord, and the results were confirmed with selected mAbs in mouse and human tissues.

Results

Antibody specificity

Binding specificities of 11 anti-ganglioside mAbs against a panel of 15 closely related synthetic and purified gangliosides used in this study (Fig. 1), as determined by ELISA (Lunn et al., 2000; Schnaar et al., 2002), are summarized in Table 1. The data for IgG-class antibodies were from prior publications (Lunn et al., 2000; Schnaar et al., 2002), except for binding to GalNAc-GD1a; only GD1a-1 and GD1a/GT1b-2b bound to this ganglioside and showed much lower affinity than to their target ganglioside. GM1-IgM showed relatively similar binding to gangliosides GM1, GD1b and asialo-GM1, which share the same Gal β3 GalNAc terminus (data not shown). For GM1-IgM, ELISA results were confirmed by TLC overlay.

Characterization of extracted gangliosides

Purified ganglioside fractions from the sensory and motor roots of human cauda equina and mouse peripheral nerves were analysed by TLC. Sialic acid staining showed no

### Table 1 Specificity of 11 anti-ganglioside mAbs against the 15 gangliosides tested in solid-phase immunoassays

<table>
<thead>
<tr>
<th>Clone</th>
<th>Ganglioside binding (relative to primary antigen)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM1-1</td>
<td>GM1 (1)</td>
</tr>
<tr>
<td>GM1-2b</td>
<td>GM1 (1)</td>
</tr>
<tr>
<td>GM1-IgM</td>
<td>GM1 (1), GD1b (1), asialo-GM1 (1)</td>
</tr>
<tr>
<td>GD1a-1</td>
<td>GD1a (1), GalNAc-GD1a (0.1), GT1aα (0.1)</td>
</tr>
<tr>
<td>GD1a-2a</td>
<td>GD1a (1), GT1aα (6)</td>
</tr>
<tr>
<td>GD1a-2b</td>
<td>GD1a (1), GT1aα (0.5)</td>
</tr>
<tr>
<td>GD1a/GT1b-2b</td>
<td>GD1a (1), GT1b (1), GT1aα (1), GalNAc-GD1a (0.2)</td>
</tr>
<tr>
<td>GD1b-1</td>
<td>GD1b (1)</td>
</tr>
<tr>
<td>GT1b-1</td>
<td>GT1b (1)</td>
</tr>
<tr>
<td>GT1b-2a</td>
<td>GT1b (1)</td>
</tr>
<tr>
<td>GT1b-2b</td>
<td>GT1b (1)</td>
</tr>
</tbody>
</table>

The ELISA titre of each mAb for each of the 15 gangliosides tested (Fig. 1) was compared with the ELISA titre of that antibody for the ganglioside used as the primary antigen in its production. The results are expressed as relative affinity. If a tested ganglioside is not listed for a particular mAb, there was no detectable binding of that mAb to that ganglioside.

3,3’-diaminobenzidine (DAB) or metal-enhanced DAB (Sigma) or Vector Red (Vector Laboratories). Data were acquired by light microscopy.

Multiple labelling

Neurofilament staining was used to label myelinated fibres, most DRG cells, ventral horn cells and neuropil. IB-4 was used as a marker for Remak bundles in spinal roots and peripheral nerves and for non-peptidergic DRG neurones. Cauda equina, sciatic nerves and DRGs were triple-labelled with individual anti-ganglioside antibodies (10–20 μg/ml), rabbit anti-neurofilament (1 : 200) and biotinylated IB-4 (5 μg/ml) by incubating overnight at 4°C. These sections were developed with specific secondary antibodies against mouse and rabbit immunoglobulins conjugated to different fluorophores (1 : 200, 1 h at ambient temperature) and IB-4 was visualized with streptavidin-conjugated CY5 (5 μg/ml). DRG neurones labelled positively with anti-neurofilament antibody were measured for diameter at the level where the nucleus was clearly identified. To prevent counting and measuring cells more than once, each cell was marked after measurement. The DRG neurones were classified as small (15–30 μm), medium (31–42 μm) or large (43–61 μm). The percentage of DRG neurones stained with a particular anti-ganglioside antibody was calculated by counting the number of ganglioside-positive DRG neurones in a given size range, dividing this number by the total number of neurofilament-positive DRG neurones in the size range, and multiplying the result by 100. This measurement was done for six representative mAbs (GM1-2b, GD1a-1, GD1a-2b, GD1b-1, GT1b-2b and GD1a/GT1b-2b), and more than 100 DRG neurones per mAb were analysed. Spinal cords were double-labelled with anti-ganglioside and anti-neurofilament antibodies. The tissue sections were mounted in anti-fade mount (Biomed, Foster City, CA, USA). Confocal microscopy was performed with an LSM510 confocal microscope system (Carl Zeiss, Jena, Germany) and images were captured using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

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Characterization of extracted gangliosides

Purified ganglioside fractions from the sensory and motor roots of human cauda equina and mouse peripheral nerves were analysed by TLC. Sialic acid staining showed no
significant quantitative differences in the expression of GD1a and other major brain gangliosides in human sensory and motor roots. However, a notable qualitative difference in human GD1a expression was detected (Fig. 2A): ventral root GD1a resolved as two bands, whereas dorsal root GD1a appeared as one major band. These bands probably carry the same GD1a saccharide, because each bound the GD1a-1, GD1a-2a, GD1a-2b and GD1a/GT1b-2b mAbs in TLC overlay (Fig. 2B). Furthermore, binding of these four mAbs was abrogated by treating the TLC plate with neuraminidase, after which anti-GM1 binding to the bands became apparent (data not shown). Binding of anti-GD1a mAbs to the minor gangliosides GalNAc-GD1a and GT1a was not detected by TLC overlay in extracted human gangliosides.

The ganglioside profile of GalNAcT−/− peripheral nerves was similar to that reported for brain (Liu et al., 1999). The peripheral nerves and spinal roots of these animals expressed only the simple gangliosides GM3 and GD3. A band of ganglioside also migrated between GM3 and GD3 and was probably O-acetyl GD3. Wild-type mice had a normal repertoire of gangliosides. The total content of ganglioside sialic acid in GalNAcT−/− and wild-type peripheral nerves was similar. TLC overlay with six mAbs (GM1-IgM, GD1a-1, GD1a-2a, GD1a/GT1b-2b, GD1b-1 and GT1b-2b) failed to show any binding to peripheral nerve gangliosides extracted from GalNAcT−/− mice, whereas these antibodies recognized specific gangliosides from wild-type nerves according to their previously determined specificities (data not shown).

Immunocytochemistry

For presentation of immunohistochemical results, mAbs are grouped according to their specificity against the four major brain gangliosides, GM1, GD1a, GD1b and GT1b. The immunostaining patterns in rats are described in detail and compared with those in human and mouse tissues. All mAbs used in this study recognized axons and neuronal structures, but failed to stain compact myelin in spinal cords, spinal roots and peripheral nerves. IB-4 was used to distinguish between ventral and dorsal roots in cauda equina sections. IB-4 stained almost all Remak bundles in the dorsal roots and peripheral nerves. Anti-calcitonin gene-related peptide and IB-4 staining co-localized in the dorsal roots and peripheral nerves (Y. Gong and K. Sheikh, unpublished observations); therefore, IB-4 alone was used as a marker of Remak bundles in all subsequent studies. With the confocal techniques used in this study, resolution was sufficient to identify Remak bundles but not sufficient for individual unmyelinated axons. The staining patterns of all mAbs are summarized in Table 2.

GD1a-binding mAbs

Four mAbs bound to GD1a: GD1a-1, GD1a-2a, GD1a-2b and GD1a/GT1b-2b.

Spinal roots and peripheral nerve

The most striking staining pattern was observed with GD1a-1, which bound preferentially to motor myelinated fibres in

![Fig. 2](image-url) Gangliosides isolated from human dorsal roots (HDR) and human ventral roots (HVR) were separated on HPTLC plates. Bovine brain gangliosides (BBG) were used as markers. (A) Resorcinol staining clearly shows a different pattern of mobility at a position corresponding to bovine brain GD1a in ventral and dorsal roots. On the basis of the literature (Ogawa-Goto et al., 1990), the ganglioside band migrating faster than GM1 is likely to be LM1 (*), a major myelin ganglioside in peripheral nerves. (B) Immunostaining with GD1a-1 (0.3 μg/ml) shows that different bands in HDR and HVR are GD1a-related.

Table 2 Summary of staining with anti-ganglioside mAbs in rat

<table>
<thead>
<tr>
<th></th>
<th>GD1a-1</th>
<th>GD1a-2a</th>
<th>GD1a-2b</th>
<th>GD1b-1</th>
<th>GT1b-1</th>
<th>GT1b-2a</th>
<th>GT1b-2b</th>
<th>GM1-1</th>
<th>GM1-2b</th>
<th>GM1-IgM</th>
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<td>Spinal cord (fixed)</td>
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<tr>
<td>Dorsal horn</td>
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<td>Ventral horn</td>
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<td>Motor fibre</td>
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<td>Sensory fibre</td>
<td>–*</td>
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<tr>
<td>Remak bundle</td>
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<td>++++</td>
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</tr>
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</table>

Staining: ++++ = strong; ++ = mild; + = minimal; – = absent. *Negative by immunofluorescence; † = some myelinated fibres in dorsal root do not stain with this antibody.
cross-sections of ventral roots and sciatic nerves (Fig. 3). This difference in binding to motor and sensory myelinated fibres was quantitative and not qualitative. For example, by increasing antibody concentration and/or using signal amplification techniques, such as ABC, we were able to show a small amount of binding in dorsal roots (Fig. 4A). Similar observations were made in human spinal roots (Fig. 5A and B), but the difference between motor and sensory fibre staining was much less obvious in mouse cauda equina.

GD1a-1 does not stain Remak bundles in rodents, but does so in human dorsal roots. The mAbs GD1a-2a and -2b strongly stained Remak bundles (Fig. 3). Staining of myelinated axons was much less intense than that of unmyelinated fibres; staining of myelinated motor axons

![Fig. 3 Fresh-frozen cross-sections of rat cauda equina triple-labelled with anti-ganglioside mAbs (green), neurofilament (red) and IB-4 (blue). Co-localization of three labels is also shown (Merged). The mAbs included in this figure are shown on the left. Bar = 20 μm.](image-url)
was more intense than that of sensory myelinated fibres. The mAb GD1α/GT1b-2b strongly stained both motor and sensory myelinated fibres and Remak bundles in dorsal roots (Fig. 3). The staining patterns with GD1α-2a, GD1α-2b and GD1α/GT1b-2b in human and wild-type mouse nerves were similar to those of rat tissue. In teased fibre preparations, all mAbs in this group stained the nodes of Ranvier and abaxonal surfaces of Schwann cells, but did not stain paranodes. Teased fibre staining with GD1α/GT1b-2b is shown in Fig. 6A.

**Spinal cord**

In rodent spinal cord sections, GD1α-1 and GD1α/GT1b-2b diffusely stained all laminae in the grey matter (Fig. 4B). The staining was very intense, more so on dorsal than on ventral horns, and distinguishing between neurones (soma) and neuropil was difficult except by double labelling with neurofilament antibodies. Furthermore, glial and extracellular matrix staining in the grey matter cannot be excluded. Axonal staining was also seen in white matter tracts. In human spinal cord sections, the distinct neuronal staining in ventral horn neurones was obvious because these neurones had shrunk away from the surrounding glia (Fig. 5C), probably because of the post-mortem interval. In rodents, GD1α-2a and -2b stained only lamina I and II in dorsal horns of the fixed spinal cords, a finding consistent with their staining of Remak bundles in dorsal roots (Fig. 4C). These mAbs stained motor neurones lightly in fresh-frozen sections, particularly in human spinal cords, but did not stain motor neurones or ventral horns in fixed sections.

**Dorsal root ganglia**

GD1α-binding mAbs differentially stained different populations of DRG neurones (Fig. 7). GD1α-1 stained only ~10% of small neurones and 20% of medium and large neurones. GD1α-2a and -2b had identical staining patterns. GD1α-2b stained >90% of small, 18% of medium and 11% of large neurones. GD1α/GT1b-2b stained >90% of DRG neurones of all sizes (Table 3). All GD1α-binding mAbs stained both IB-4-positive and -negative small DRG neurones.

**GM1-binding mAbs**

The three mAbs in this group (GM1-1, GM1-2b and GM1-IgM) stained myelinated axons in both motor and sensory spinal roots. GM1-2b and -IgM also stained Remak bundles in dorsal spinal roots (Fig. 3). Interestingly, some medium to large myelinated axons in dorsal roots were not stained by GM1-2b. Teased fibre preparations showed discrete staining of the nodes of Ranvier and abaxonal Schwann cell surfaces (Fig. 6B). The intensity of abaxonal Schwann cell staining was strongest for the GM1 mAbs. The nodal staining pattern with these antibodies differed from the pattern with cholera toxin in that there was no paranodal staining. All mAbs stained the grey matter in the spinal cord diffusely, including ventral motor neurones, with similar staining patterns among rat, human and wild-type mouse tissues. GM1-2b stained 85% of small, 45% of medium and 60% of large DRG neurones (Fig. 7 and Table 3). This antibody stained both IB-4-positive and -negative small DRG neurones. GM1-IgM and GM1-1 also stained small, medium and large DRG neurones. Staining with these two mAbs was not quantified.
GD1b-binding mAb
GD1b-1 stained both motor and sensory myelinated axons in spinal roots (Fig. 3), but did not stain Remak bundles. Nodes of Ranvier and abaxonal Schwann cell membranes were stained in teased fibre preparations. In spinal cord sections, grey matter, including motor neurone soma in ventral horns, was stained diffusely. GD1b-1 stained >90% of medium and large DRG neurones and <20% of small DRG neurones (Fig. 7 and Table 3). Most small neurones stained by this mAb were IB-4-negative. Rat, human and mouse tissues had similar staining patterns.

GT1b-binding mAbs
All three mAbs in this group (GT1b-1, GT1b-2a and GT1b-2b) stained myelinated motor and sensory axons in spinal roots and peripheral nerves (Fig. 3). Remak bundles were more strongly stained with GT1b-2a and GT1b-2b than with GT1b-1. All three mAbs stained the nodes of Ranvier and abaxonal Schwann cell plasmalemma in teased fibre preparations. Diffuse grey matter and motor neurone staining was seen on spinal cord sections. All three populations of DRG neurones were stained by the mAbs in this group (Fig. 7). Only the staining of DRG neurones with GT1b-2b was quantified, and it showed that 85% of small and >90% of medium and large DRG neurones were labelled with this mAb (Table 3). Rat, human and mouse tissues showed similar staining patterns.

Control studies
No staining was seen when primary antibodies were omitted, except in mouse tissue, where some endoneurial (including minimal axon staining), perineurial and epineurial staining was seen with secondary (anti-mouse immunoglobulin) antibodies. Staining of the three tested mAbs (GD1a-1, GD1b-1 and GM1-1) was significantly decreased after neuraminidase treatment, with maximal decrease in fixed spinal cord floating sections, probably because neuraminidase had better penetration.

Solvent extraction
Treatment with chloroform–methanol either completely abolished or significantly decreased the binding of all mAbs except GD1a/GT1b-2b and GM1-IgM, which retained significant binding (Fig. 8). The staining on GalNACT/−/− tissues was consistent with results of solvent treatment. GD1a/GT1b-2b and GM1-IgM antibodies clearly stained myelinated axons in the GalNACT/−/− tissues (Fig. 8E and F) and significant binding was retained after solvent extraction, although the
amount was decreased. No other mAbs stained tissues from knockout animals. Notably, low-level background (secondary antibody) axonal staining was detected in the fresh-frozen peripheral nerves of transgenic mice, and therefore the possibility of low-level antibody binding to tissues from knockout animals cannot be excluded definitively.

Discussion
Specific high-affinity IgG anti-ganglioside mouse antibodies, similar in their binding specificities to those seen in Guillain–Barré syndrome, had distinctive staining patterns in the PNS in three species: human, rat and mouse. The staining patterns in all three species were similar. The major finding of this
study is that some IgG anti-ganglioside mAbs selectively stain certain neuronal and nerve fibre populations. These differences in staining are not explained by ganglioside content in the motor and sensory nerves. Rather, this observation provides strong support for the concept that specific anti-ganglioside antibodies associated with various neuropathic syndromes may selectively recognize and injure particular nerve fibre and neuronal populations. Other notable findings are that all mAbs used in the present study stained axons and neurones preferentially and that two anti-ganglioside mAbs with broad specificity cross-reacted with epitopes that were likely to be peripheral nerve glycoproteins, because they were not susceptible to solvent extraction and were present in GalNAcT knockout mice.

Selective staining patterns
Our results showed that mAb GD1a-1 clearly had preferential binding to motor nerve fibres compared with sensory fibres. To a much lesser extent, mAbs GD1a-2a and -2b displayed the same relative specificity. In contrast, GD1a/GT1b-2b displayed no differences between motor and sensory nerve fibre staining. The basis of differential recognition of motor and sensory nerves by GD1a-binding mAbs remains unresolved. Consistent with previous reports (Svennerholm et al., 1992, 1994; Ogawa-Goto and Abe, 1998), biochemical data show no significant quantitative differences in GD1a content in ventral and dorsal roots of humans. Therefore, quantitative differences in GD1a content in ventral and dorsal roots cannot account for the differences observed by immunohistochemistry. Furthermore, GD1a-binding mAbs did not show more binding to extracted ventral than to dorsal root gangliosides by TLC immuno-overlay. However, the TLC mobilities of ventral and dorsal root GD1a species were distinct, suggesting that there were differences in the fatty acid chain length in the ceramide portion of motor and sensory nerve GD1a. This difference might possibly affect the conformation of the oligosaccharides of GD1a in their native membrane, which could explain the differences in monoclonal reactivity observed by immunohistochemistry.

An alternative possibility is that these mAbs recognize another ganglioside in the motor nerves. GalNAc-GD1a expression in the peripheral nerves has been reported (Ilyas et al., 1988b), and one report suggests that this ganglioside may only be expressed by motor neurones and axons (Yoshino, 1997). Although this would provide one potential explanation for preferential motor axon staining by GD1a-1, this mAb has approximately 10-fold greater binding to GD1a than GalNAc-GD1a, and this explanation would not be applicable to mAbs GD1a-2a and -2b, which show no binding to GalNAc-GD1a. Furthermore, TLC immuno-overlay studies using GD1a-1 on gangliosides extracted from human motor and sensory nerves showed binding to GD1a only, suggesting that the expression of GalNAc-GD1a is very low. Similar findings were also seen in the rat motor and sensory nerves (Y. Tagawa and K. Sheikh, unpublished observations). These observations would argue against the hypothesis of GalNAc-GD1a as target antigen.

The possibility that another minor ganglioside/glycolipid antigen is recognized preferentially in the motor fibres cannot be excluded. Our results are consistent with a recent report of preferential motor nerve staining by serum from an AMAN patient with IgG reactivity to GD1a and GalNAc-GD1a (Lugaresi et al., 1997; De Angelis et al., 2001). Whatever the basis of preferential motor axon binding may be, these findings provide one explanation for the selective motor nerve injury seen in the AMAN variant of Guillain–Barré syndrome.

Our studies provide two examples of selective staining of different populations of DRG neurones. GD1a-2a and -2b appear to be specific markers of almost all adult small DRG neurones. These two mAbs have the strongest binding to Remak bundles in the dorsal spinal roots and peripheral nerves, to small DRG neurones, and to the central projection fibres to the lamina I and II in the dorsal horn of the spinal cord, and they recognize both peptidergic (IB-4-negative) and non-peptidergic (IB-4-positive) small DRG neurones. The staining intensity of motor and sensory myelinated fibres, medium and large DRG neurones, and ventral motor neurones is several-fold less than that of the Remak bundles and small DRG neurones. GD1a-2a bind better to the closely related minor ganglioside GT1a than to GD1a; however, GT1a, a very minor component of mammalian gangliosides (approximately one-thousandth the concentration of GD1a), is reported to be specific for cholinergic neurones and is therefore unlikely to be contributing significantly to the binding we detected. In contrast to GD1a-binding antibodies, GD1b-1 did not stain a majority of small DRG neurones and Remak bundles, and instead is a marker for most adult medium and large DRG neurones. This is interesting since

<table>
<thead>
<tr>
<th>DRG size (μm)</th>
<th>GD1a-1 (n = 132)*</th>
<th>GD1a-2b (n = 124)</th>
<th>GD1a/GT1b-2b (n = 109)</th>
<th>GM1-2b (n = 134)</th>
<th>GD1b-1 (n = 137)</th>
<th>GT1b-2b (n = 128)</th>
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<tr>
<td>15–30</td>
<td>8/70 (11)</td>
<td>54/59 (91)</td>
<td>47/50 (94)</td>
<td>65/76 (85)</td>
<td>12/69 (17)</td>
<td>57/67 (85)</td>
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<td>31–42</td>
<td>7/31 (22)</td>
<td>7/38 (18)</td>
<td>25/27 (92)</td>
<td>15/33 (45)</td>
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<td>43–60</td>
<td>7/31 (22)</td>
<td>3/27 (11)</td>
<td>32/32 (100)</td>
<td>15/25 (60)</td>
<td>29/29 (100)</td>
<td>28/28 (100)</td>
</tr>
</tbody>
</table>

Data are number of positive DRG neurones/total number counted for that size range (percentage of positive cells). *n = total DRG neurones analysed per mAb.

Table 3 Summary of DRG neurone staining with six selected mAbs

Localization of gangliosides 2501
small DRG neurones bound GT1b-specific mAbs, so they must synthesize GD1b as a precursor to GT1b. Therefore, GD1b is either inaccessible in small DRG neurones or is metabolized efficiently to GT1b.

**Preferential axonal staining**
All antibodies used in this study preferentially stained neurones, neuropil and axons. Compact myelin was not stained significantly in either peripheral nerves or spinal cords. Although GM1 and other complex gangliosides are present in the myelin fractions, as indicated by chemical studies (Ogawa-Goto *et al.*, 1990; Svennerholm *et al.*, 1994), these gangliosides are apparently not accessible by immunohistochemical techniques used in the present study. This observation is consistent with our previous findings in which cholera toxin did not stain compact myelin significantly (Sheikh *et al.*, 1999). A commercially available anti-galactocerebroside mAb (Chemicon, Temecula, CA, USA) also did not stain compact myelin significantly (Y. Gong and K. Sheikh, unpublished observations). Teased fibre preparations showed that all mAbs stained the axonal surface of Schwann cells. This staining was relatively higher for GM1 mAbs. Paranodal staining on teased fibre preparations, which has been reported with most anti-ganglioside antibodies (Chiba *et al.*, 1993; Kusunoki *et al.*, 1993, 1997; Molander...
et al., 1997; Paparounas et al., 1999), was not seen with any mAbs used in this study. This absence of staining is unlikely to be an artefact of the method used, because cholera toxin did stain paranodes in these preparations. These observations do not clarify why some anti-ganglioside antibodies are associated with predominant axonal injury and others with myelin injury. If anti-ganglioside antibodies are involved in peripheral nerve demyelination, then it is likely that surface expression of glycolipid antigens on Schwann cells is sufficient for antibody binding and the activation of downstream events that can lead to demyelination.

Cross-reaction of anti-ganglioside mAbs with protein(s)
Most of the anti-ganglioside mAbs reported here stained gangliosides exclusively, as evidenced by diminished staining after solvent extraction and lack of staining of tissues from GalNAcT–/– mice. However, two mAbs with broad specificities, GM1-IgM and GD1a/GT1b-2b, retained significant staining after solvent extraction and bound to neural tissue from GalNAcT–/– mice, indicating that the antigen recognized in the knockout mice is not a glycolipid. We tentatively conclude that GM1-IgM and GD1a/GT1b-2b cross-react with proteins in the nerves, perhaps by binding to (Galβ3 GalNAc) (Shuman et al., 1983; Rieger et al., 1986; Hoffinan et al., 1988; Crossin et al., 1989) and [NeuAcα3 Galβ3 (NeuAc α6) GalNAc] determinants on O-linked glycoproteins, respectively. This finding may have significance for the pathogenetic effects of similar human antibodies. Further work is needed to determine the structures of the apparent non-lipid cross-reactive species for these two mAbs, and their biological and/or pathophysiological relevance.

Implications for anti-ganglioside antibody-mediated nerve damage
Motor neuropathies
Anti-GD1a antibodies were reported in some patients with Guillain–Barré syndrome (Ilyas et al., 1988a), and subsequent case reports and small case series reported the presence of anti-GD1a antibodies in the motor and/or axonal variants of Guillain–Barré syndrome or paraproteinemic neuropathy (Yuki et al., 1993a; Carpo et al., 1996; Lugas et al., 1997; Hao et al., 1998; Odaka et al., 1999). We reported that in northern China IgG anti-GD1a antibodies were closely associated with the AMAN variant of Guillain–Barré syndrome (Ho et al., 1999). Recently, GalNAc-GD1a, a minor ganglioside related to GD1a, was implicated as a target antigen in motor variants of Guillain–Barré syndrome (Yuki et al., 1996, 1999; Hao et al., 1999; Kaida et al., 2000). Anti-GM1 antibodies are seen in several clinical settings; in particular those of the IgG class are seen in some patients with AMAN and demyelinating variants of Guillain–Barré syndrome (Ho et al., 1995; Rees et al., 1995; Kuwabara et al., 1998). IgM anti-GM1 antibodies are present in most sera from patients with multifocal motor neuropathy (Pestronk et al., 1988; Pestronk and Choksi 1997).

A vexing issue in this context is the lack of a satisfactory explanation for the selective motor axon injury that occurs in AMAN. The only previously reported differences in the ganglioside content (including GM1 and GD1a) of motor and sensory nerves are those in the fatty acid chain lengths in the ceramide portion of sensory and motor nerve gangliosides (Ogawa-Goto et al., 1990). Previous localization studies, including our own, mostly in the context of anti-GM1 antibodies, were limited by the use of either low-affinity (usually IgM) anti-ganglioside antibodies or high-affinity bacterial toxins or lectins (Corbo et al., 1993; Apostolski et al., 1994; Molander et al., 1997; Sheikh et al., 1999). These studies failed to show preferential binding to relevant gangliosides in motor fibres.

The results of the present study support the concept that selective anti-ganglioside antibody binding to motor fibres is one mechanism for the selective motor nerve injury seen in motor autoimmune neuropathies. The AMAN variant of Guillain–Barré syndrome associated with anti-GD1a antibodies best implies such a sequence of pathogenetic events. The differences in staining of motor nerve compared with sensory nerve fibres were not clearly obvious for GM1. In accordance with the results of previous studies and our current findings, it is likely that other factors, such as antibody accessibility and differential neuronal/nerve fibre susceptibility to injury, may also contribute to the clinicopathological phenotype. Our results call attention to the importance of immunohistochemical studies with appropriate reagents/antibodies to address such issues.

Sensory neuropathies
The clinical phenotype associated with anti-GD1b antibodies is sensory ataxic neuropathies. This association has been observed in both chronic and acute settings, including Guillain–Barré syndrome. Kusunoki and colleagues induced a sensory ataxic phenotype in rabbits by immunization with ganglioside GD1b (Kusunoki et al., 1996, 1999). Pathological examination of these rabbits showed degeneration of the large sensory neurones (Kusunoki et al., 1996). Immunohistochemical studies showed staining of the large sensory neurones with GD1b antibodies (Kusunoki et al., 1997). The present study shows that GD1b-1 mAb stained >95% of large and medium DRG neurones and <20% of small DRG neurones. These results support the concept that anti-GD1b antibodies may cause selective injury to large sensory neurones.

In contrast, GD1a-2a and -2b selectively stained almost all small DRG neurones and Remak bundles. The staining pattern seen with these two mAbs could be relevant to
non-metabolic small-fibre neuropathies, the vast majority of which are idiopathic; the clinical and pathological features imply injury exclusively to small sensory neurones or to their processes contained in the Remak bundles. Rarely, small fibre neuropathies with acute or subacute onset similar to Guillain–Barré syndrome have been described (Holland et al., 1998). In the light of current findings, it is attractive to speculate that a small subset of these cases, particularly those with acute onset, are autoimmune and that anti-ganglioside antibodies should be considered as injurious agents.

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