to binding autoantibodies, NMJ gangliosides act aberrantly as target molecules for clostridial neurotoxins (Schiavo et al., 2000; Bullens et al., 2002).

Antibody binding to target membranes results in complement activation with membrane attack complex (MAC) pore formation (Koski et al., 1987; Acosta et al., 1996). Our studies on anti-disialosyl ganglioside Ab binding to presynaptic NMJ gangliosides indicate that uncontrolled calcium influx through MAC pores triggers massive neurotransmitter release with electrophysiological failure and calpain-mediated disintegration of the presynaptic axon (Plomp et al., 1999; Bullens et al., 2000; O'Hanlon et al., 2001, 2003; Halstead et al., 2004).

Evidence that anti-GD1a antibodies play a pathogenic role in AMAN is limited (Zhang et al., 2004). The difficulty in generating a mouse model of AMAN is likely attributable to a combination of the poor immunogenicity of gangliosides and the low level of GD1a in mouse motor axons accessible to circulating Abs. Overcoming immunological tolerance has been made possible by using ganglioside-deficient, glycosyl transferase knock-out mice (Lunn et al., 2000; Bowes et al., 2002). However, because these mice no longer contain any target antigen, they are resistant to development of autoimmune disease when immunized with gangliosides, despite having high levels of circulating antibody. Here we overcame these factors in a two-stage immunization protocol. First, we cloned anti-GD1a monoclonal Abs (mAbs) from GD1a-deficient (i.e., immunologically nontolerant) β-1,4-*N*-acetylgalactosaminyl transferase knock-out (*GalNAcT*$^{-/-}$) mice immunized with GD1a-containing *C. jejuni* LOS. Second, we applied these anti-GD1a mAbs with added complement to distal motor nerve preparations from α-2,8-sialyl transferase II knock-out (*GD3s*$^{-/-}$) mice that express abnormally high levels of GD1a (see Fig. 1) (Kawai et al., 2001; Okada et al., 2002) and looked for pathophysiological effects.

## Materials and Methods

### *Mice*

Male *GD3s*$^{-/-}$ (overexpressing the "a-series" gangliosides) (Okada et al., 2002), *GalNAcT*$^{-/-}$ (expressing only GM3, GD3, and GT3) (Takamiya et al., 1996), and wild-type (WT) mice were used at 6–8 weeks of age. A simplified scheme of ganglioside biosynthesis with the O-series omitted for clarity is shown in Figure 1. Ganglioside nomenclature is according to IUPAC-IUB Joint Commission on Biochemical Nomenclature (1998) and Svennerholm (1994). All mice were on a C57BL/6-CBA background. Control observations indicate that anti-ganglioside antibodies have the same effects in C57BL/6 mice as in a mixed C57BL/6-CBA background (S. K. Halstead, P. D. Humphreys, and J. A. Goodfellow, unpublished data). All animal experiments were performed according to United Kingdom and Dutch laws and Glasgow and Leiden University guidelines (Glasgow University, government license number PPL60/3096; Leiden University, government license number DEC 01055).

### *Anti-GD1a-LOS mAbs*

mAbs were cloned from *GalNAcT*$^{-/-}$ mice immunized with *C. jejuni* HS:19 or HS:4 strains expressing LOS structures identical to GD1a (Aspinall et al., 1994; Bowes et al., 2002). HS:3 was used as an irrelevant *C. jejuni* isolate devoid of ganglioside-like structures (Aspinall et al., 1995). Fusions were screened against GD1a by ELISA using previously reported protocols (Goodyear et al., 1999). mAbs were characterized for isotype, subclass, and ganglioside-binding profiles (GM1, GM2, GM3, GD1a, GD1b, GD3, GT1b, and GQ1b) using standard ELISA and thin-layer chromatography-based techniques (Goodyear et al., 1999). mAbs were purified from tissue culture supernatants using HiTrap Protein A/G affinity columns (Amersham Biosciences, Bucks, UK), concentrated to 2 mg/ml, and stored at −70°C. Relative levels of mAb reactivity to a panel of gangliosides were determined from ELISA titration curves over antibody concentrations of $10^{-1}$ to $10^{-6}$ mg/ml and were expressed as the reciprocal of the antibody concentration that gave half-maximal binding (Paterson et al., 1995). The anti-GD1a mAbs cloned from mice immunized with GD1a conjugated to keyhole limpet hemocyanin (GD1a-KLH conjugate) have been reported previously (Lunn et al., 2000; Schnaar et al., 2002).

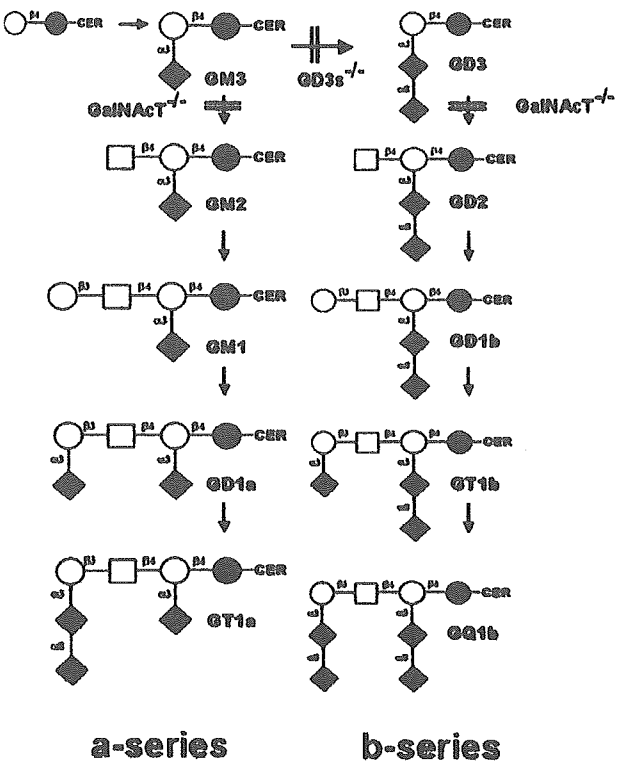


**Figure 1.** Abbreviated ganglioside biosynthetic pathway. In the *GalNAcT*$^{-/-}$ mice, disruption of the β-1,4-*N*-acetylgalactosaminyl transferase gene (Unigene name, Galgt1) results in the absence of all of the complex gangliosides beyond GM3 and GD3 (Takamiya et al., 1996). In the *GD3s*$^{-/-}$ mice, disruption of the α-2,8-sialyl transferase II gene (Unigene name, Siat8a) results in the absence of b-series gangliosides and a compensatory overexpression of a-series gangliosides, including GD1a (Kawai et al., 2001; Okada et al., 2002). WT mice express varying levels of all gangliosides shown. Nomenclature is according to the IUPAC-IUB Joint Commission on Biochemical Nomenclature (1998) and Svennerholm (1994). CER, Ceramide; ○, galactose; □, *N*-acetylgalactosamine; ●, glucose; ◆, *N*-acetylneuraminic acid/sialic acid. Carbon–carbon linkages are also shown.

### *Antibodies, complement, and immunochemicals*

Mouse anti-GD1a antisera were obtained from *GalNAcT*$^{-/-}$ mice immunized with GD1a-KLH emulsified in complete Freund's adjuvant. GD1a-KLH conjugate was prepared by ozonolysis of GD1a and reductive amination using sodium cyanoborohydride (Helling et al., 1994). Preimmune and hyperimmune sera were tested for anti-GD1a antibody titers by ELISA. Six human anti-GD1a-positive sera (titers from 1:11,000 to 1:23,000) were obtained from acute-phase AMAN patients (subject to local ethical committee approval and guidelines). Mouse and human anti-GD1a sera were depleted of complement by heat inactivation at 56°C for 30 min and diluted 1:2 in Ringer's solution before experiments. Normal human serum (NHS) as a source of heterologous complement was taken from a single healthy donor and stored in multiple aliquots at −70°C to maintain inter-experimental consistency.

The following primary and secondary antibodies were used: rabbit anti-neurofilament (NF) antibody 1211 (Affinity BioReagents, Exeter, UK); mouse IgG1 anti-NF antibody 1217 (Cambridge Bioscience, Cambridge, UK); FITC goat anti-mouse IgG and CY5 or FITC goat anti-rabbit IgG, and CY5 goat anti-mouse IgG1 (Southern Biotech Associates, Birmingham, AL); FITC-goat anti-human C3c, mouse IgG2a anti-

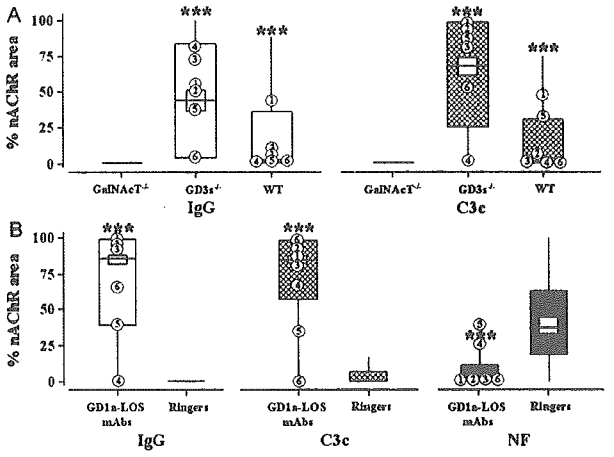


**Figure 2.** Effects of anti-GD1a-LOS antibodies. Immunohistological analysis of anti-GD1a-LOS antibody binding, complement fixation, and destruction of NF is shown. *A*, Topical application to diaphragm. "% nAChR area" represents the area of the NMJ stained with TxR-BTx that is covered with fluorescent anti-IgG or anti-C3c signal. These values were calculated as described in Materials and Methods and expressed as the median (horizontal line), interquartile range (large box), and 1.5× the interquartile range (vertical line) of pooled data. IgG (white) or C3c (hatched) levels at NMJs in sections of WT, *GD3s*$^{-/-}$, and *GalNAcT*$^{-/-}$ diaphragm are shown. Highest levels of IgG and C3c are seen in the *GD3s*$^{-/-}$ mouse. *B*, *Ex vivo GD3s*$^{-/-}$ hemidiaphragm preparations. IgG (white), C3c (hatched), and NF (black) levels at NMJs were calculated and expressed as in *A*. Ringer's solution or anti-GD1a-LOS antibody (100 μg/ml in Ringer's solution) was applied, followed by a source of complement. Anti-GD1a-LOS mAbs bind to the NMJ, fix complement, and cause an NF loss in the *GD3s*$^{-/-}$ mouse. ***$p < 0.001$ compared with Ringer's control solution. Median values of individual anti-GD1a-LOS mAbs are plotted as follows: 1, MOG-32; 2, MOG-34; 3, MOG-35; 4, TBG-1; 5, TBG-2; 6, TBG-3.

human MAC, and rabbit anti-cow S100 (Dako, Glostrup, Denmark); and FITC goat anti-mouse IgG2a (Bethyl Laboratories, Montgomery, TX).

Immunochemicals and other reagents were as follows: Texas-Red-labeled α-bungarotoxin (TxR-BTx) and Alexa Fluor BTx (Molecular Probes, Eugene, OR; citifluor antifade medium (Citifluor, Canterbury, UK); ethidium homodimer 1 (EthD) (Molecular Probes); and goat serum, Triton X-100, and L-lysine (Sigma-Aldrich, Dorset, UK).

### *Nerve muscle preparations*

Male *GalNAcT*$^{-/-}$, *GD3s*$^{-/-}$, and WT mice (6–8 weeks old) were killed by $CO_2$ inhalation. Diaphragm or triangularis sterni muscles were dissected out onto a Sylgard-lined base of a dish containing pregassed Ringer's solution (O'Hanlon et al., 2001) at room temperature (RT) (20–22°C). All nerve muscle preparations were exposed to anti-GD1a mAbs/sera (or Ringer's control solution) for 2.5 h at 32°C and then 30 min at 4°C (to accentuate antibody binding) and 10 min at RT unless otherwise stated. Diaphragm was rinsed and then exposed to 40% NHS (or Ringer's control solution) for 1 h at RT. For immunohistology or electron microscopy, a 1 cm square section of tissue was snap frozen on dry ice or fixed in 2.5% paraformaldehyde/2.5% glutaraldehyde, respectively. For analysis of perisynaptic Schwann cell (pSC) integrity, a 1 cm square section was further incubated in EthD for 1 h at RT and then fixed in 0.1% paraformaldehyde, as described previously (Halstead et al., 2004).

### *Immunohistology*

*Topical binding studies of anti-GD1a antibodies on diaphragm sections.* Diaphragm cryostat sections (8 μm) from *GalNAcT*$^{-/-}$, *GD3s*$^{-/-}$, and WT mice were placed onto 3-aminopropyltrithoxysilane (APES)-coated slides. Sections were then treated with anti-GD1a mAbs (50 μg/ml in PBS) for 2 h at 4°C with or without 4% NHS for an additional 1 h at RT. Anti-GD1a antibodies or human C3c were detected with FITC goat anti-mouse IgG (1:300) or FITC goat anti-human C3c (1:200;1 h, 4°C). TxR-BTx (1:1000) was included in the secondary antibody solution.

*Diaphragm nerve muscle preparations.* Eight and 15 μm cryostat sections of snap-frozen diaphragm tissues previously exposed *ex vivo* to anti-GD1a antibodies/sera and complement (as described above) were placed onto APES-coated slides. Anti-GD1a IgG or human C3c were detected with the FITC goat anti-mouse IgG (1 h, 4°C) or the FITC goat anti-human C3c (1 h, 4°C). TxR-BTx was included in the secondary antibody solution. For detection of NF, sections were first treated with TxR-BTx, fixed in ethanol (20 min, −20°C), and then exposed to rabbit anti-NF 1211 antibody (1:200 in 1% goat serum and 0.1% Triton X-100, overnight, 4°C), followed by FITC goat anti-rabbit IgG (1 h, 4°C). Tissue prepared for analysis of pSC integrity with EthD was sectioned at 20 μm and treated with Alexa Fluor BTx (1:1000; 1 h, RT).

*Triangularis sterni nerve muscle preparations.* For whole-mount illustrations of anti-GD1a antibody binding, live *ex vivo* triangularis sterni preparations were incubated with anti-GD1a mAb MOG-35 as above, fixed in 4% paraformaldehyde (20 min, RT), and rinsed in PBS, then 0.1 M glycine, and then PBS (10 min each, 4°C). Tissue was incubated overnight at 4°C in mouse anti-NF 1217 (IgG1) or rabbit anti-cow S100 (both at 1:200; in 1% goat serum, 0.4 M lysine, and 0.5% Triton X-100). Primary antibodies were detected with FITC anti-mouse IgG2b and either CY5 anti-mouse IgG1 or CY5 anti-rabbit IgG (all 1:300), and nicotinic acetylcholine receptors (nAChRs) were detected with TxR-BTx (1:1000; 3.5 h, 4°C).

### *Electrophysiological studies*

Electrophysiological recordings were made from left and right hemidiaphragms as described previously (Plomp et al., 1999). Briefly, intracellular recordings of miniature endplate postsynaptic potentials (MEPPs) were made using standard recording equipment. Randomly within the preparation, muscle fibers were impaled near the NMJ with a 10–20 MΩ glass microelectrode filled with 3 M KCl. MEPP frequency was measured as follows: (1) before addition of anti-GD1a antibody/serum; (2) after their addition (100 μg/ml for MOG-35 anti-GD1a mAb; 1:2 in Ringer's solution for complement-inactivated AMAN serum) for 3 h at 33°C (and 30 min at 4°C in case of AMAN serum); and (3) during subsequent 1 h period of 40% NHS (at 20–22°C). If high MEPP frequency occurred that led to spontaneous fiber twitches (Plomp et al., 1999), μ-conotoxin-GIIIB (1.5 μM; Scientific Marketing, Barnet, UK) was added to block muscle $Na^+$ channels, as well as contractions. In some diaphragm preparations, EPPs were measured under these conditions. Tissue was then washed in Ringer's medium and snap frozen for immunohistology. Signals were digitized and stored for later, off-line analysis. Ten to 25 NMJs were sampled from each experimental condition, and each experimental condition was repeated three times.

### *Imaging and image analysis*

All fluorescence images were acquired using a Bio-Rad (Hemel Hempstead, UK) MRC1024 or Zeiss (Oberkochen, Germany) Pascal confocal microscope. Complete NMJs in whole-mount triangularis sterni or 30 μm diaphragm sections were reconstructed using Voxx three-dimensional voxel-based rendering software (Clendenon et al., 2002). Electron micrographs of NMJs were prepared, viewed, and photographed as described previously (O'Hanlon et al., 2001).

For quantitative analysis, the area of the nAChR signal covered by IgG, C3c, or NF is expressed as percentage of nAChR area. Nonparametric data pooled from three to five experimental preparations are presented as the median (horizontal line), 95% confidence intervals (small box), interquartile range (large box), and 1.5× interquartile range (vertical lines). Individual data points lying outside 1.5× interquartile range are not shown in the figures for clarity but were included in all calculations and statistical analysis. Median values of individual mAbs or antisera are indicated in the figures by numbered circles. For topical staining, 436–503 endplates were examined per experimental condition and, for *ex vivo* preparations, 529–953 endplates. Mann–Whitney mean rank test was used to compare possible statistical differences between groups. *$0.05 > p > 0.005$; **$0.005 > p > 0.001$; ***$p < 0.001$.

## Results

### Ganglioside and LOS binding patterns of anti-GD1a mAbs

Six IgG monoclonal anti-GD1a antibodies raised in *GalNAcT*$^{-/-}$ mice immunized with *C. jejuni* LOS were cloned for this study.

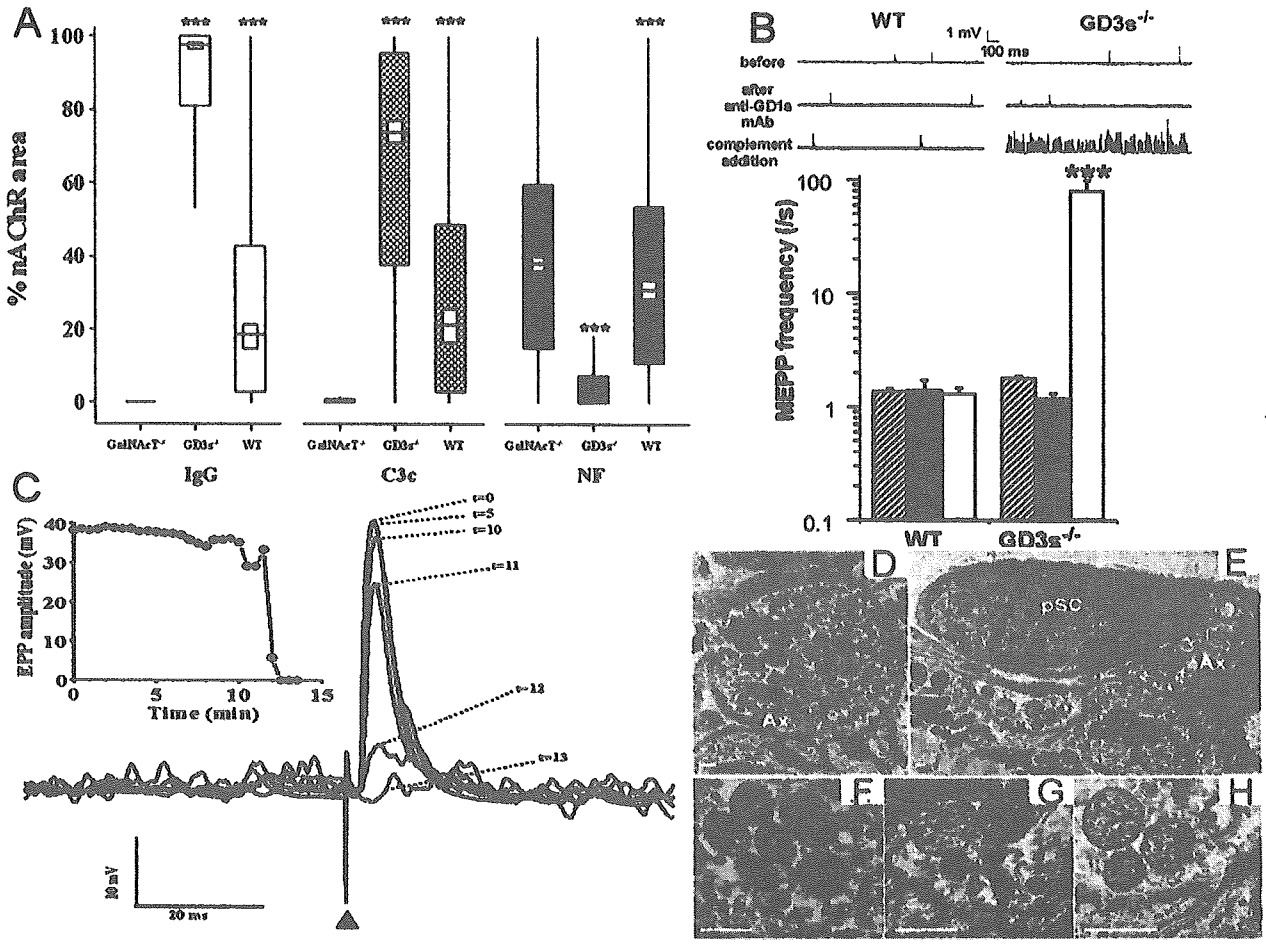


**Figure 3.** *Ex vivo* injury by MOG-35 is GD1a dependent. *A*, The anti-GD1a-monospecific IgG MOG-35 was able to bind to *ex vivo* NMJs, fix complement, and cause a loss of NF in a GD1a-dependent manner. IgG, C3c, and NF levels are expressed as in Figure 2*B*. White, black, and hatched rectangles show IgG, C3c, and NF levels, respectively, for each genotype calculated as described in Materials and Methods. *GD3s*$^{-/-}$ nerve terminals are more susceptible to antibody binding, complement fixation, and NF loss than WT or *GalNAcT* $^{-/-}$ nerve terminals. ***$p < 0.001$. *GD3s*$^{-/-}$ was compared with WT. WT was compared with *GalNAcT* $^{-/-}$. *B*, MOG-35 causes a massive increase in MEPP frequency in the presence of complement at the *ex vivo GD3s*$^{-/-}$ NMJ. MEPP recordings before (hatched bars) and after (black bars) MOG-35 addition demonstrate that the mAb alone has no effect on NMJ transmission. Subsequent addition of 40% NHS (white bar) causes a massive increase in asynchronous spontaneous neurotransmitter release at *GD3s*$^{-/-}$ but not WT NMJs (sample traces shown). IgG, C3c, and NF levels of the preparations that were electrophysiologically tested were equivalent to those shown in *A*. ***$p < 0.001$ compared with WT. *C*, Example of neurotransmission failure at *GD3s*$^{-/-}$ NMJs induced by MOG-35 and subsequent addition of 40% NHS. The phrenic nerve of a *GD3s*$^{-/-}$ diaphragm nerve–muscle preparation was stimulated once every 30 s over a 13 min monitoring period, in the presence of $\mu$-conotoxin-GIIIB to prevent muscle action potentials. EPPs were recorded at an NMJ in which MEPP frequency was very high (>100/s, visible at the baseline of traces). EPPs were normalized to −75 mV and superimposed. The EPP decreased with time after complement addition and became blocked. This did not occur at WT or *GalNAcT* $^{-/-}$ nerve terminals and did not occur without the anti-GD1a mAb. *D–H*, Ultrastructure of MOG-35- or Ringer's solution-treated *GD3s*$^{-/-}$ diaphragm preparations. *D*, *F*, Control *GD3s*$^{-/-}$ diaphragm preparations show normal NMJ morphology, including an abundance of synaptic vesicles, electron-dense mitochondria, and healthy pSCs. *E*, *G*, *H*, MOG-35- and complement-treated *GD3s*$^{-/-}$ diaphragm preparations show grossly abnormal morphological features, including a generally swollen and electron-lucent appearance, depletion of synaptic vesicles, and electron-lucent mitochondria. pSCs retain a normal appearance. Scale bars, 0.25 $\mu$m. Ax, Axon terminal.

MOG-32, MOG-34, and MOG-35 (all IgG2b) were raised against HS:19 LOS. TBG-1 (IgG2b) and TBG-2 and TBG-3 (both IgG3) were raised against HS:4 LOS. All antibodies reacted with the immunizing LOS from the GBS-associated *C. jejuni* strain HS:19 or HS:4 but not to irrelevant LOS from the uncomplicated enteritis-associated strain HS:3 (data not shown). With respect to ganglioside reactivity, all mAbs bound to GD1a with half-maximal binding values in the $10^3$ range, i.e., at a concentration of ~1 $\mu$g/ml on the midpoint of the titration curve. All six mAbs also bound weakly to GT1b by 1–2 log-fold lower magnitudes. GT1b shares the terminal NeuAc($\alpha$2–3)Gal-GalNAc trisaccharide epitope with GD1a. Two mAbs also reacted very weakly with GM1 (TBG-2 and MOG-34) and one with GD1b (MOG-32). Other gangliosides screened (GM2, GM3, GD1b, GD3, and GQ1b) were not bound.

### Levels of neural GD1a determine the extent of binding of anti-GD1a-LOS mAbs

We first tested the ability of anti-GD1a-LOS antibodies to bind and fix complement during topical application at the NMJs of diaphragm sections from mice with different levels of expression of GD1a (i.e., *GD3s*$^{-/-}$, high GD1a; WT, normal GD1a; *GalNAcT*$^{-/-}$, no GD1a). Topical application of anti-GD1a-LOS mAbs and 4% NHS as a source of complement resulted in IgG and complement (measured as the intermediate component, C3c) deposition at NMJs (Fig. 2*A*). The NMJs from the *GD3s*$^{-/-}$ mouse showed significantly higher levels of IgG and C3c deposition than WT mice, and the *GalNAcT*$^{-/-}$ mouse that lacks any GD1a (or GT1b) showed no detectable levels of IgG or C3c. The *GalNAcT*$^{-/-}$ mouse acts as an ideal negative control because it allows each antibody to act as its own concentration and isotype-

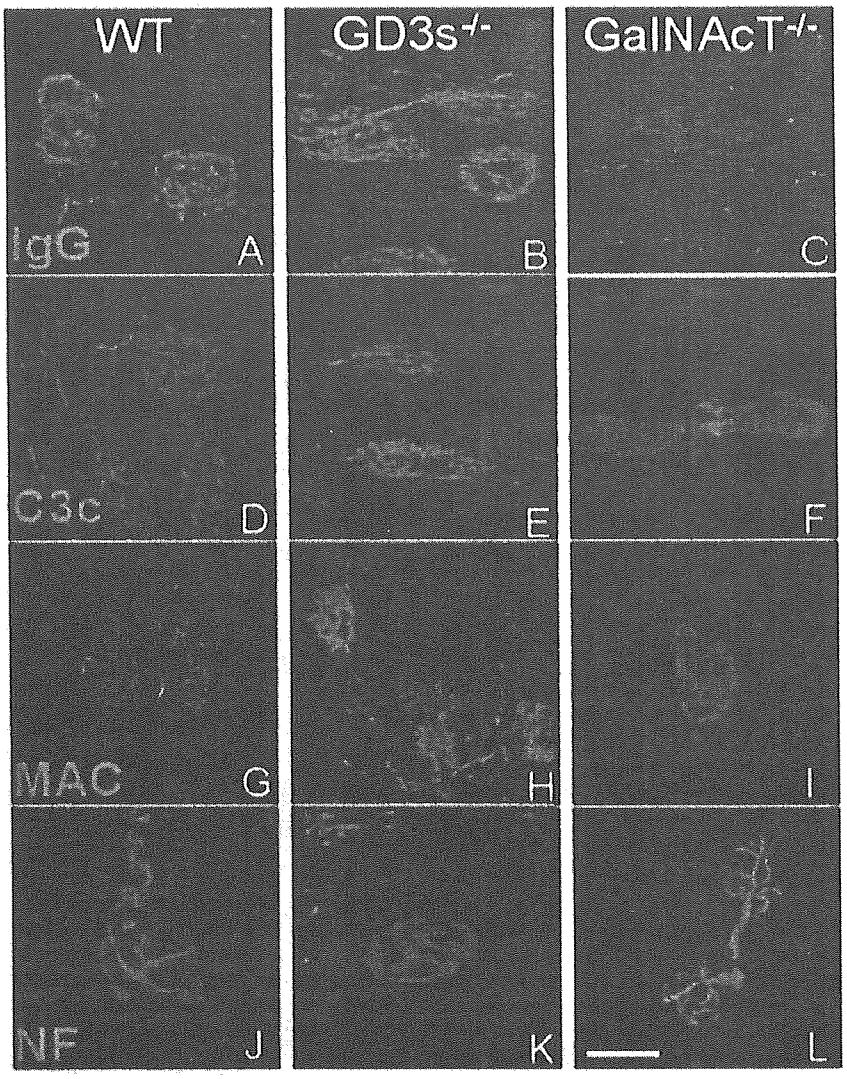


**Figure 4.** Neurofilament loss is antibody, complement, and GD1a dependent. Reconstructed confocal images of MOG-35- and complement-treated WT, $GD3s^{-/-}$, and $GalNAcT^{-/-}$ diaphragm NMJs are shown. TxR-BTx staining is shown in red in all panels. Green indicates the following: *A–C*, mouse IgG; *D–F*, human C3c; *G–I*, human MAC; *J–L*, NF. Images were acquired and reconstructed under identical settings for each genotype. WT NMJs showed low levels of IgG (*A*), C3c (*D*), and MAC (*G*) and high levels of NF (*J*). $GD3s^{-/-}$ endplates showed high levels of IgG (*B*), C3c (*E*), and MAC (*H*) and low levels of NF (*K*). $GalNAcT^{-/-}$ NMJs showed essentially no IgG (*C*), C3c (*F*), or MAC (*I*) and high levels of NF (*L*). Scale bar, 20 μm.

matched control and confirms that the specificity of effect is ganglioside dependent rather than being related to nonspecific interactions or cross-reactive glycoconjugate effects. These data indicate that the anti-GD1a-LOS antibodies bind to the NMJ in a GD1a concentration-dependent manner and suggest that the $GD3s^{-/-}$ mouse should be more vulnerable to anti-GD1a antibody-mediated injury than normal WT mice.

### Anti-GD1a-LOS mAb and complement cause NMJ injury and paralysis *ex vivo* in $GD3s^{-/-}$ mice

In view of its predicted increase in sensitivity to injury, the $GD3s^{-/-}$ mouse was then used to screen for pathological effects on terminal axons of all six of the anti-GD1a antibodies raised by immunization with GD1a bearing LOS. Axonal injury was monitored by loss of NF immunoreactivity, the main axonal cytoskeletal protein in nerve (O'Hanlon et al., 2001). Exposure of $GD3s^{-/-}$ NMJs *ex vivo* to anti-GD1a mAbs resulted in IgG binding, C3c deposition as expected from Figure 2*A*, and concomitant NF loss (Fig. 2*B*). Thus, all six anti-GD1a mAbs examined induced axonal injury.

To demonstrate the importance of the level of GD1a in rendering the NMJ liable to axonal injury, the effects of one mAb, MOG-35, were examined in WT (normal GD1a), $GD3s^{-/-}$ (high GD1a), and $GalNAcT^{-/-}$ (no GD1a) diaphragm preparations. These data clearly show that GD1a levels influences the magnitude of NF loss as a measure of axonal injury and that this correlates with the level of bound IgG and subsequent C3c deposition (Fig. 3*A*); thus, the higher levels of IgG and C3c seen at $GD3s^{-/-}$ NMJs translate into a greater injury (low NF signal) than that seen in WT or $GalNAcT^{-/-}$ mice. (Median values for tissue treated with Ringer's solution alone were as follows: IgG, <5%; C3c, <11%; NF, >34% for all genotypes. Therefore, although the anti-GD1a mAb MOG-35 caused a statistically significant loss of NF in the WT compared with $GalNAcT^{-/-}$, it was a very minor loss that may not be biologically significant.)

Electrophysiological examination of MOG-35 plus complement-treated WT, $GD3s^{-/-}$, and $GalNAcT^{-/-}$ diaphragm preparations showed a massive increase in MEPP frequency at the $GD3s^{-/-}$ mouse NMJs (Fig. 3*B*), eventually leading to block of synaptic transmission as measured by evoked EPPs (Fig. 3*C*). WT NMJs were unaffected (Fig. 3*B*), as were $GalNAcT^{-/-}$ NMJs (data not shown). Subsequent immunohistological analysis of these electrophysiological preparations demonstrated similar IgG, C3c, and NF levels to those in Figure 3*A* (data not shown).

Previously, we showed that Miller-Fisher syndrome (MFS)-related anti-disialosyl antibodies could bind to pSCs, activate complement, and lead to pSC death as monitored by an EthD uptake assay (Halstead et al., 2004). For the anti-GD1a-LOS mAb MOG-35 up to a concentration of 500 μg/ml, there was no significant increase in the proportion of NMJs showing EthD-positive pSCs compared with Ringer's solution-treated preparations (data not shown). This indicates that pSCs are not targeted by anti-GD1a Abs, even in GD1a-enriched $GD3s^{-/-}$ mice.

NMJ tissues were examined for ultrastructural evidence of injury, in accordance with our previous studies (O'Hanlon et al., 2001; Halstead et al., 2004).

Compared with controls (Fig. 3*D,F*), MOG-35- and complement-treated preparations showed highly abnormal axonal ultrastructure (vesicle depletion, mitochondrial swelling, and electron lucency) at the NMJ (Fig. 3*E,G,H*). pSCs appeared morphologically normal (Fig. 3*E*).

### Localization of IgG and complement products at MOG-35-exposed NMJs

To determine the immunolocalization of IgG and complement deposits within nerve terminals, serial confocal images of MOG-35-treated diaphragm NMJs were reconstructed. In the preparations from *GalNAcT*$^{-/-}$ mice that lack GD1a, there was no detectable IgG, C3c, or MAC deposition, and there were normal levels of NF (Fig. 4*C,F,I,L*). In WT mice, low-level IgG, C3c, and MAC deposits were observed with near-normal NF signal (Fig. 4*A,D,G,J*). In the *GD3s*$^{-/-}$ preparations, levels of IgG, C3c, and MAC were very highly aligned with the gutters of the NMJ, and little or no NF signal was present over the NMJ and distal axon (Fig. 4*B,E,H,K*).

To improve the resolution of MOG-35 localization at the NMJ, we used three-dimensional reconstructions of whole-mount triangularis sterni neuromuscular preparations from *GD3s*$^{-/-}$ mice (Fig. 5). These studies were conducted in the absence of complement, thereby avoiding tissue injury and allowing NF staining to delineate the axon. MOG-35 immunoreactivity appears exclusively localized to the axolemma, enveloping neurofilament bundles (Fig. 5, blue in *A*, *D*, *I–K*) that sit on the bungarotoxin-labeled gutters beneath and distinct from the pSC (labeled with the Schwann cell marker S100 in blue in *E*, *H*, *L–N*). Parallel preparations treated with Ringer's solution instead of MOG-35 and imaged and reconstructed under the same settings showed no IgG immunoreactivity (data not shown).

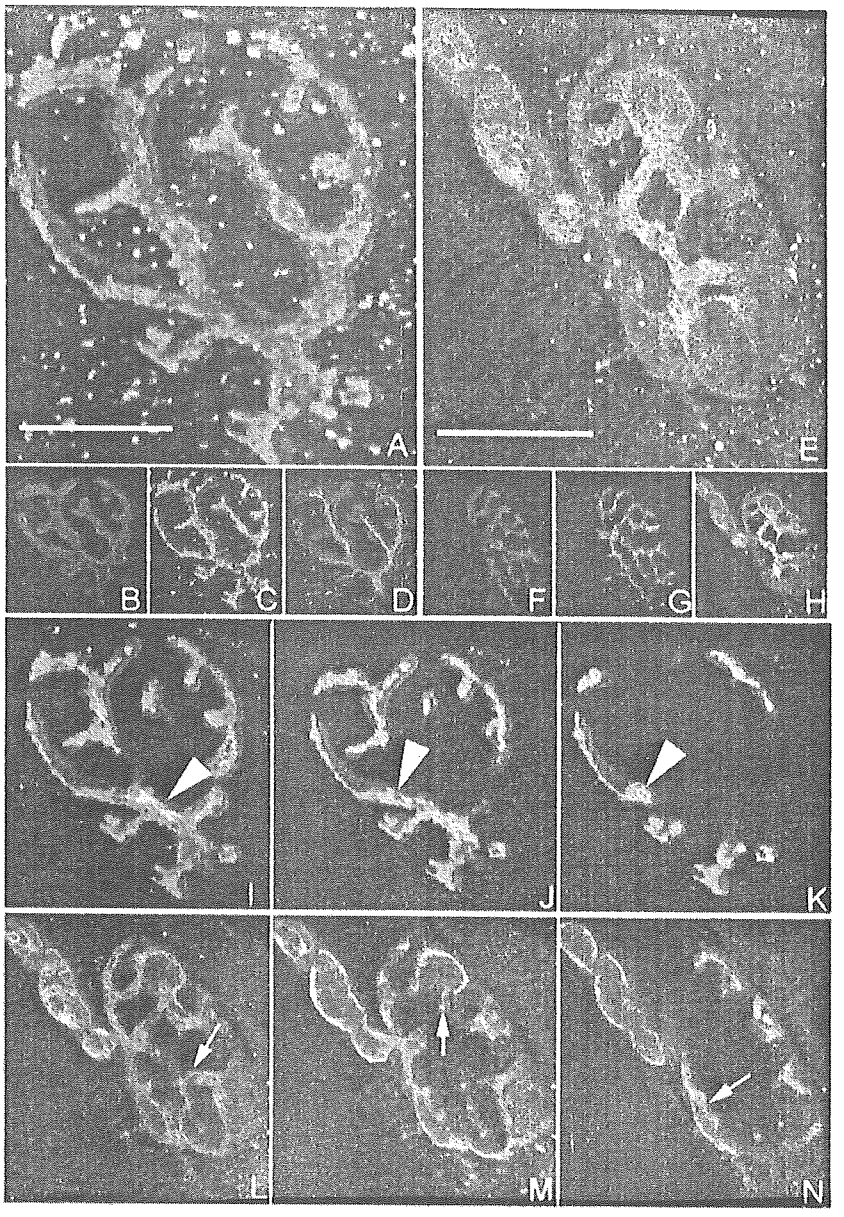


**Figure 5.** Localization of anti-GD1a-LOS mAb at the NMJ. Immunofluorescent localization of MOG-35 at *GD3s*$^{-/-}$ triangularis sterni NMJs is shown. Reconstructed confocal images of MOG-35-treated *GD3s*$^{-/-}$ preparations showing the localization of GD1a (MOG-35 staining), AChRs (TxR-BTx staining), neurofilament (anti-NF antibody staining), and perisynaptic Schwann cells (S100 staining). *C*, MOG-35 (green) was localized over gutters of the BTx (red) signal (*B* shows BTx alone). *D*, NF (blue) shows a similar localization over the BTx signal. *A*, A merged image of *C* and *D* indicated that the MOG-35 signal "wrapped" most of the NF signal. *G*, Another example of MOG-35 (green) localized over the BTx signal (red) (*F* shows BTx alone). *H*, The Schwann cell marker S100 (blue) labeled the pSC and terminal myelinating Schwann cell at this NMJ. *E*, A merged image of *G* and *H* indicated that the majority of MOG-35 staining was "underneath" the S100 signal, whereas the abaxonal pSC membrane was almost unstained. *I–K*, Serial sections through *A*. *L–N*, Serial sections through *E*. Arrowheads in *I–K* highlight MOG-35 staining that "wrapped" the NF signal. Arrows in *L–N* highlight MOG-35 staining that occurred essentially "underneath" the S100 staining. This is consistent with a primarily axonal localization, an adaxonal pSC membrane localization of MOG-35, or both. Scale bars, 10 μm.

### Anti-GD1a-positive human and mouse sera bind to terminal motor axons in a GD1a-dependent manner and can cause an NF loss *ex vivo*

Anti-GD1a-KLH mAbs and anti-GD1a-KLH hyperimmune sera obtained by immunizing *GalNAcT*$^{-/-}$ mice with GD1a-KLH conjugate had identical pathological effects to anti-GD1a-LOS mAbs at WT, *GD3s*$^{-/-}$, and *GalNAcT*$^{-/-}$ diaphragm NMJs (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). This demonstrates that anti-GD1a antibodies arising through alternative routes to preceding infection with an LOS-bearing bacterial antigen are similarly pathogenic.

To establish the relevance for human neuropathy of the anti-GD1a mAb data, we investigated the effects of acute-phase anti-GD1a IgG-positive sera from AMAN patients ($n$ = 6; anti-GD1a titers from 1:11,000 to 1:23,000), alongside control sera from multiple sclerosis cases ($n$ = 3; negative for anti-GD1a antibodies). One AMAN sera was also examined in an *ex vivo* electrophysiological study. In the presence of a source of complement, this latter serum caused a massive increase in MEPP frequency and eventual transmission block in a large proportion of the *GD3s*$^{-/-}$ NMJs *ex vivo*, whereas the MEPP frequency increase was very minor at WT NMJs and no transmission block was observed (Fig. 6*A*). Immunohistological analysis of these preparations (Fig. 6*B*) revealed that *GD3s*$^{-/-}$ NMJs had elevated levels of C3c and reduced NF immunoreactivity compared with WT.

Human anti-GD1a-specific IgG cannot be identified because it is masked by nonspecific background staining attributable to polyclonal IgG in both the serum and in the NHS used as a source of complement. This same serum sample and five others were then tested, using immunohistological methods, for pathogenic effects *ex vivo* in the *GD3s*$^{-/-}$ hemi-diaphragm preparation (Fig. 6*C*). Four of the six AMAN sera caused a significant loss of NF but spared the pSCs. These data show that human anti-GD1a AMAN serum displays the same pathological potential as mouse anti-GD1a mAbs and antiserum.

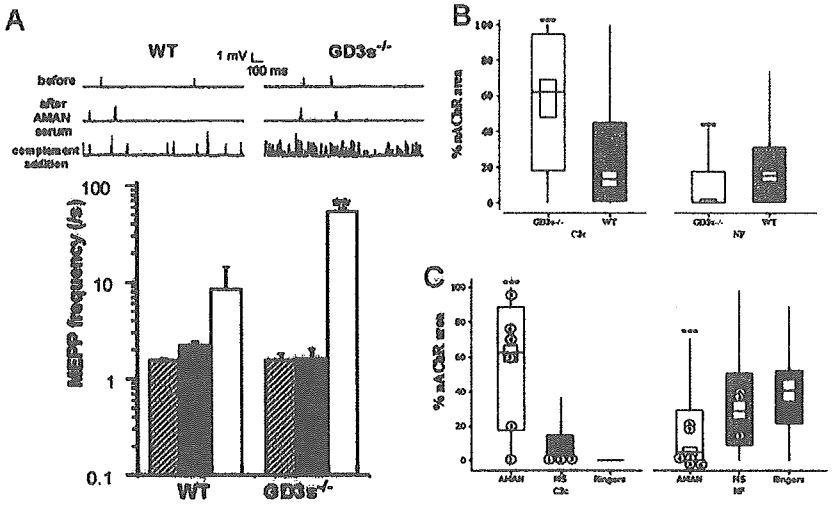


**Figure 6.** Effects of human AMAN serum. Addition of AMAN serum followed by a source of complement resulted in a massive increase in asynchronous uniquantal ACh release (measured as MEPPs) at *GD3s*$^{-/-}$ diaphragm NMJs *ex vivo*. *A*, Electrophysiological recordings of MEPPs before (hatched bars) and after (black bars) complement-inactivated AMAN serum addition demonstrated that the serum alone had no effect. Subsequent addition of 40% NHS (white bar) caused a massive increase in MEPP frequency (sample traces shown). $^{**}p < 0.005$ compared with WT. *B*, Immunohistological investigation of NMJs from these preparations revealed that *GD3s*$^{-/-}$ (white box) NMJs from AMAN serum-treated preparations had elevated levels of C3c and reduced levels of NF compared with WT (hatched box) NMJs. The median value, the interquartile range, and 1.5× the interquartile range of C3c and NF data are expressed as described in Materials and Methods. $^{***}p < 0.001$ compared with WT. *C*, Six human anti-GD1a-positive AMAN sera were tested for pathogenic effects using immunohistological methods in the *GD3s*$^{-/-}$ mouse as described in Materials and Methods. Parallel preparations treated with multiple sclerosis (MS) sera or Ringer's solution served as controls. Compared with both multiple sclerosis- (hatched box) or Ringer's solution- (black box) treated preparations, the AMAN sera (white box) led to elevated levels of C3c deposition and decreased levels of NF at the NMJ. The level of complement fixation varied considerably between the AMAN samples, but four of the six caused a very large reduction in NF (median of 0 for samples 1, 3, 4, and 6), whereas the other two caused a minor loss in NF.

## Discussion

We found that anti-GD1a antibodies cloned from mice immunized with GD1a-like epitopes on *C. jejuni* LOS cause acute destructive changes at mouse NMJs *ex vivo* in a complement- and GD1a-dependent manner. Human anti-GD1a antibody-positive AMAN serum and mouse sera containing polyclonal anti-GD1a antibodies or GD1a mAbs raised from GD1a-KLH immunization all are capable of inducing these effects. Besides illustrating that anti-GD1a antibodies can be generated through the molecular mimicry mechanism, these data demonstrate the capacity of anti-GD1a antibodies to play a direct role in mediating distal motor axon-specific pathology in a model of human disease. This is also the first direct demonstration, to our knowledge, that GD1a is present at motor nerve terminals.

Our data suggest that a defect at the NMJ may contribute to the paralytic clinical features of AMAN, although recognizing this does not preclude injury at other sites along the motor axon (Hafer-Macko et al., 1996; De Angelis et al., 2001; Sheikh et al., 2004). Nevertheless, the absence of a blood–nerve barrier at the motor nerve terminal does render the site especially vulnerable to injury through circulating factors, including autoantibodies and toxins. Several studies have implicated neurotransmission failure as a contributing factor in some GBS patients (Ho et al., 1997; Spaans et al., 2003), including MFS patients (Uncini and Lugaresi, 1999; Wirguin et al., 2002), and in AMAN patients in whom reduced safety factor at the nerve terminal was implicated as an important factor in conduction block (Kuwabara et al., 2003). Although the exact role that NMJ injury may play in the clinical syndromes remains undetermined, this model nevertheless provides an interesting system for studying the acute-phase pathogenic effects of anti-GD1a antibodies and for studying the neuronal and glial response to the degenerative and regenerative phases of autoimmune distal axonal injury.

Remarkably, we found that NMJs from normal mice were resistant to anti-GD1a-mediated injury. Thus, a key strategy was the use of *GD3s*$^{-/-}$ mice that express higher levels of GD1a than WT mice to act as the tissue target for anti-GD1a Abs. Conversely, because *GalNAcT*$^{-/-}$ mice do not express GD1a, they are ideal for raising anti-GD1a antibodies because they do not exhibit immunological tolerance (Lunn et al., 2000; Bowes et al., 2002). However, they are not susceptible to developing anti-GD1a-mediated disease because they lack target antigen. We thus manipulated these two mouse strains in a "cross-immunization" paradigm in which mice lacking GD1a (*GalNAcT*$^{-/-}$) were used to induce a potent anti-GD1a immune response, and mice expressing high levels of GD1a (*GD3s*$^{-/-}$) were used as vehicles to provide an antigen-rich target for the pathophysiological effects of that response. Had we used WT mice throughout this study, we would have encountered both poor humoral immune responses to GD1a and poor levels of tissue injury, thus failing to induce an immunopathological phenotype. This underlines the power of experimentally controlling the level of target antigen, a notion that has been more extremely applied to other models of autoimmune disease precipitated by neoself antigen expression (Cornet et al., 2001). This concept has clear clinical relevance, because, in MFS, the vulnerability of the extraocular nerves to anti-GQ1b antibody-mediated injury is attributed to their higher levels of GQ1b compared with motor nerves innervating the limb (Chiba et al., 1997). The level of expression of GD1a in different regions of the human peripheral nervous system, and the possibility that individuals vary in their ganglioside composition, has not been examined but could provide support for this hypothesis.

Anti-GD1a antibodies did not induce any deleterious effects on pSCs, in contrast to previously studied MFS-associated anti-GQ1b antibodies (Halstead et al., 2004). This underlines the capacity of anti-GD1a antibodies to mediate axon-specific pathology. Electron microscopy and vital dye staining revealed normal pSC integrity and ultrastructure. Moreover, the whole-mount three-dimensional reconstructions of NMJs clearly showed selective axonal anti-GD1a antibody binding. This parallels the selec-

tive axonal injury seen in AMAN patients, in which motor axons are damaged whereas Schwann cells are spared (Hafer-Macko et al., 1996; De Angelis et al., 2001).

Not all anti-GD1a antibody-positive AMAN patients show evidence of preceding infection with an LOS-bearing bacterial agent. Viral agents are hypothesized to lead to anti-ganglioside response via presentation of host ganglioside during viral budding from infected cells (Evans and Webb, 1986; Ang et al., 2000). The characteristics and pathological properties of our anti-GD1a antibodies and antisera arising in response to GD1a-KLH immunization were very similar to those seen in response to LOS immunization. This strongly supports the notion that mouse and human anti-GD1a antibodies arising through different mechanisms are similarly capable of inducing highly selective motor axonal injury.

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## Does *Campylobacter jejuni* infection elicit "demyelinating" Guillain–Barré syndrome?

**To the Editor:** We read with interest the article by Kuwabara et al.[1] Their series includes 159 consecutive patients with Guillain–Barré syndrome (GBS). There was evidence of recent *Campylobacter jejuni* infection in 22 patients, 16 of them being electrophysiologically classified as acute motor axonal neuropathy (AMAN), five as acute inflammatory demyelinating polyneuropathy (AIDP), and the remaining one as unclassified. Since these AIDP patients showed transient slowing of nerve conduction mimicking demyelination, the authors concluded that *C. jejuni* infection does not appear to elicit AIDP.

We reported a patient aged 67 years with fulminant GBS who died 18 days after onset.[2] One week before admission, he had an upper respiratory infection. Three serial electrophysiological examinations revealed universal nerve inexcitability. Using reported techniques,[3] both IgM and IgG anti-*C. jejuni* antibodies were detected at 1/40 serum dilution (positive as of 1/10); stool culture for *C. jejuni* was not done. There were no increased titers of IgM or IgG antiganglioside antibodies (GM1, asialo-GM1, GM2, GM3, GD1a, GD1b, GD3, GT1b and GQ1b). Autopsy showed extensive inflammatory demyelination of spinal roots and a variable combination of axonal degeneration and demyelination in peripheral nerve trunks including femoral, median, ulnar and sural nerves. This is a prototypic example of a severe GBS case combining primary demyelination and axonal degeneration secondary to inflammation.

One outstanding pathological finding was radicular paranodal demyelination (see figure 2C).[2] We argued that this finding concurred with the suggestion that an immune reaction to *C. jejuni* could mediate antibody- and complement-mediated reactions directed to target epitopes in the paranodal region and periaxonal space, recruiting macrophages, and ultimately leading to fiber degeneration.[2]

Unlike China and Japan where AMAN predominates, European epidemiological surveys have demonstrated that AIDP is the most prevalent form of GBS, axonal variants representing around 6% of cases.[4] In another study conducted in Britain,[5] *C. jejuni* infection was associated with both AIDP and AMAN. As stated by Kuwabara et al., host susceptibility factors could account for differences between Eastern and Western countries.

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**Reply from the Authors:** We thank Drs. Berciano and Illa for their interest in our paper,[1] which suggests that *C. jejuni* infection is not likely to elicit the demyelinating form GBS—AIDP—in Japan.

Berciano et al.[2] reported a case of severe GBS with serologic evidence of recent *C. jejuni* infection and an autopsy showing AIDP pathology. We agree with the comment that primary severe demyelination and secondary axonal degeneration were responsible for inexcitable nerves in that patient. However, we think that only positive serology for anti-*C. jejuni* assay is not sufficient evidence for preceding *C. jejuni* infection. There are no standards for serologic testing for this bacterium with regard to antigens and cutoff values for the positivity, and actually, the sensitivity and specificity of serologic assays varies considerably among laboratories.[6]

The specificity of our assay is 88%, and in combination with a clinical history of a definite diarrheal illness in our study, the strict criteria could reduce the false-positive cases. Moreover, 91% of our 22 anti-*C. jejuni*-positive patients were antiganglioside positive. In the case reported by Berciano et al., an antecedent event was upper respiratory infection, antiganglioside antibodies were negative, and stool culture was not done. It is unlikely that his patient suffered *C. jejuni* infection.

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# Patterns and serial changes in electrodiagnostic abnormalities of axonal Guillain–Barré syndrome

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**Abstract**—*Background:* In Guillain–Barré syndrome (GBS), anti-ganglioside antibodies are strongly associated with the acute motor axonal neuropathy (AMAN) form, but there are also cases of the demyelinating form of GBS (acute inflammatory demyelinating polyneuropathy [AIDP]) with anti-ganglioside antibodies. *Objective:* To elucidate the patterns and sequential changes in electrodiagnostic abnormalities of anti-ganglioside-positive GBS. *Methods:* Detailed serial electrodiagnostic findings were reviewed for 51 patients with GBS. Anti-ganglioside antibodies were measured by ELISA. *Results:* Antibodies to GM1, GM1b, GD1a, or GalNAc-GD1a were present in 25 patients. Of these, 12 (48%) showed the AMAN pattern, 5 (20%) the AIDP pattern, and 3 (12%) isolated F-wave absence in the first examination. All five patients with the AIDP pattern showed prolonged distal latencies, but three eventually showed the AMAN pattern or rapid normalization. The remaining two still had similarly prolonged distal latencies in weeks 4 to 6, but the serial changes were distinct from those in the anti-ganglioside-negative AIDP patients who showed progressive increases in distal latencies over 2 months after onset. *Conclusions:* Besides the simple axonal degeneration pattern, patients with anti-ganglioside-positive Guillain–Barré syndrome can show transient conduction slowing/block in the distal or proximal nerve segments, mimicking demyelination, but anti-ganglioside antibodies do not appear to be associated with acute inflammatory demyelinating polyneuropathy.

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Clinical, electrophysiologic, and pathologic studies indicate that Guillain–Barré syndrome (GBS) includes primary axonal and demyelinating forms: acute motor axonal neuropathy (AMAN) and acute inflammatory demyelinating polyneuropathy (AIDP).[1-4] The frequency of the GBS subtypes varies among countries: AMAN has been found in only 7% of patients with GBS studied in England,[5] 3% in a multicenter study of 11 Western countries,[6] and 65% in northern China.[4] In Western countries, GBS is usually caused by AIDP, and the patterns and sequential changes of the nerve conduction abnormalities in AIDP have been well described.[6-8] In contrast, little is known about the serial electrodiagnostic abnormalities in patients with axonal GBS.

Previous studies have shown that anti-ganglioside antibodies are frequently found in the sera from patients with AMAN; in particular, IgG antibodies to the gangliosides GM1, GM1b, GD1a, and GalNAc-GD1a may play an important role in the pathophysiology of AMAN.[9-17] However, the relationship between anti-ganglioside antibodies and neurophysiology is still debated. In northern China, positive anti-GM1 antibody serology was found for 10 (48%) of 21 AMAN patients and for 4 (33%) of 12 AIDP patients.[4] Our previous study showed that 4 (12%) of 34 anti-GM1-positive patients were diagnosed with AIDP.[15] These results raise the possibility that anti-ganglioside antibodies are associated with both AMAN and AIDP. In addition, AMAN was originally characterized by axonal degeneration of the motor fibers, showing simple reduction of the compound muscle action potentials (CMAPs), but some patients with the electrodiagnosis of AMAN have shown rapid clinical improvement accompanied by restoration of the distal CMAP amplitude, suggesting a conduction block in the distal nerve segments.[10,18] Therefore, the electrophysiologic classification during the early phase of the illness can change in a considerable number of AMAN patients.[15]

To elucidate the patterns of nerve conduction abnormalities in the early phase and their sequential changes in anti-ganglioside-positive GBS, we reviewed serial electrodiagnostic studies and compared the results with those of anti-ganglioside-negative typical AIDP.

Additional material related to this article can be found on the *Neurology* Web site. Go to www.neurology.org and scroll down the Table of Contents for the March 8 issue to find the title link for this article.

**Methods.** *Patients.* This study included 51 patients with GBS who underwent the first electrodiagnostic studies performed within 14 days of onset and participated in two or more follow-up studies within the first 6 weeks of onset. All fulfilled the clinical

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criteria for GBS.[19] The patients' disabilities were evaluated on the Hughes functional grading scale.[20]

*Electrophysiology.* Nerve conduction studies were done with a Nicolet Viking IV EMG machine (Nicolet Biomedical Japan, Tokyo) on the day of admission and sequentially for up to 6 months after onset. Motor nerve conduction studies were made of the median, ulnar, tibial, and peroneal nerves, including F-wave analyses, and anti-dromic sensory conduction studies were performed in the median, ulnar, and sural nerves. The patients were classified as having the AMAN or AIDP pattern based on the electrodiagnostic criteria of the motor nerve conduction study results.[4]

When patients had one of the following findings in two or more nerves during the first 2 weeks of illness, they were classified as having AIDP: 1) conduction velocity <90% of lower limit of normal if amplitude is >50% of the lower limit of normal, <85% if amplitude is < 50% of lower limit of normal; or 2) distal latency >110% of upper limit of normal if amplitude is normal, >120% of upper limit of normal if the amplitude is less than lower limit of normal; or 3) evidence of unequivocal temporal dispersion; or 4) F-response latency >120% of normal. When patients had no evidence of demyelination as defined for AIDP and had decrease in CMAP to <80% of lower limit of normal in two or more nerves, patients were classified as having AMAN. Isolated F-wave absence was defined as the F-wave persistency <20% with normal peripheral nerve conduction.[14] When the nerve conduction parameters did not meet the criteria for AIDP and AMAN but were outside the normal range, the patients were classified as having "minor abnormalities." Acute motor sensory axonal neuropathy (AMSAN) was defined as the presence of AMAN pattern in motor nerve studies and an amplitude reduction <50% of the normal limits of the sensory nerve action potentials (SNAPs) in two or more nerves.[21] Normal control data were obtained from 101 healthy subjects. For distal latency, CMAP duration, conduction velocity, and F-wave latency, we defined a value outside 2.5 SD from the mean as abnormal. For the CMAP or SNAP amplitude, we defined abnormality as present if the amplitude fell below 2.5 SD of the mean of the logarithmically transformed amplitudes of the controls.[10,15]

*Anti-ganglioside antibody testing.* The serum samples were tested for the presence of IgG antibodies to GM1, GM1b, GD1a, and GalNAc-GD1a by ELISA, as described elsewhere.[22] The antibody titer (1:*X*) was the highest serum dilution at which the optical density at 492 nm was ≥0.1. Serum was considered positive when the titer was ≥1:500. The antibodies measured have been reported to be closely associated with the AMAN electrodiagnosis.[15,17]

*Statistical analysis.* Differences in proportions were tested with the $\chi^2$ or Fisher exact test and differences in medians with the Mann–Whitney *U* test. $p < 0.05$ was considered significant.

**Results.** *Clinical profiles.* Twenty-five patients had IgG antibodies to GM1, GM1b, GD1a, or GalNAc-GD1a. Table 1 compares the clinical profiles of patients with and without these antibodies. Anti-ganglioside-positive patients more frequently had preceding gastroenteritis and less frequently upper respiratory tract infections. There were no significant differences in the disabilities between the two groups at the peak of illness. The anti-ganglioside-positive patients had less frequent facial palsy and sensory symptoms. In the anti-ganglioside-positive group, sensory symptoms were found in 10 of 25 (40%). However, 9 of the 10 had mild paresthesia/pins-and-needles in the distal limbs, and only the remaining 1 patient showed a decrease in sensations. Seventy-three percent of the anti-ganglioside-negative patients had sensory symptoms, and most of them showed decreases in vibratory, touch, or pain sensation dominantly in the distal limbs.

*Anti-ganglioside antibodies and electrodiagnostic findings.* Table 2 shows the electrodiagnostic findings in the first and follow-up studies done in weeks 3 to 6 in the patients with anti-ganglioside antibodies and those without them. In the first studies, there were significantly higher percentages of patients in the anti-ganglioside-positive group who showed the AMAN pattern compared with the anti-ganglioside-negative group, but there were no significant differences in the frequency of AIDP between the two groups. Three (12%) anti-ganglioside-positive patients had F-wave absence as an isolated nerve conduction abnormality. None had a conduction block (>20% reduction of CMAP amplitude between the stimulation at the wrist and elbow of the median or ulnar nerves). In the follow-up studies done in weeks 3 to 6, the percentage of AMAN patients increased in the anti-ganglioside-positive group, and the AIDP pattern increased in the anti-ganglioside-negative group. Five of the 25 anti-ganglioside-positive patients and 10 of the

***Table 1*** *Clinical features of patients with Guillain–Barré syndrome*

| | IgG antibody to GM1, GM1b, GD1a, or GalNAc-GD1a | | |
|---|---|---|---|
| | Positive, n = 25 | Negative, n = 26 | *p* |
| Age, mean ± SD; y | 42.2 ± 17.3 | 41.3 ± 20.5 | NS |
| Sex, M/F | 15/10 | 15/11 | NS |
| Antecedent infection, no. (%) | | | |
| Gastroenteritis | 17 (68) | 3 (12) | <0.001 |
| URTI | 5 (20) | 14 (54) | 0.01 |
| None/others | 3 (12) | 9 (35) | NS |
| Hughes grade at nadir, median/mean/range | 4.0/3.1/1–5 | 3.5/3.6/1–6 | NS |
| Clinical symptom, no. (%) | | | |
| Facial palsy | 8 (32) | 19 (73) | 0.004 |
| Bulbar palsy | 7 (28) | 14 (54) | NS |
| Sensory symptoms | 10 (40) | 19 (73) | 0.02 |

URTI = upper respiratory tract infection.

***Table 2*** *Initial and final electrodiagnostic findings in patients with Guillain–Barré syndrome*

| | IgG antibody to GM1, GM1b, GD1a, or GalNAc-GD1a | | |
|---|---|---|---|
| | Positive, n = 25 | Negative, n = 26 | *p* |
| First study, no. (%) | | | |
| AIDP pattern | 5 (20) | 11 (42) | NS |
| AMAN pattern | 12 (48) | 1 (4) | <0.001 |
| Absent F waves | 3 (12) | 4 (15) | NS |
| Minor abnormalities | 5 (20) | 10 (38) | NS |
| Follow-up study in wk 3–6, no. (%) | | | |
| AIDP pattern | 2 (8) | 14 (54) | <0.001 |
| AMAN pattern | 17 (68) | 3 (12) | <0.001 |
| Absent F waves | 0 (0) | 0 (0) | NS |
| Minor abnormalities | 4 (16) | 9 (35) | NS |
| Normal | 2 (8) | 0 (0) | NS |

AIDP = acute inflammatory demyelinating polyneuropathy; AMAN = acute motor axonal neuropathy; Absent F waves = absence of F waves as an isolated abnormality.

26 anti-ganglioside-negative patients showed minor abnormalities in the first studies. These patients had less severe disability; the mean Hughes grade was 2.6 in the ganglioside-positive group and 3.1 in the ganglioside-negative group. These patients were considered to have milder disease in each subgroup of GBS.

*Sequential electrodiagnosis in patients with anti-ganglioside antibodies.* Sequential electrodiagnosis of the 25 patients with anti-ganglioside antibodies are summarized in table E-1 on the *Neurology* Web site at to www.neurology.org. In the first studies done in week 1 or 2, 12 (48%; nos. 1 to 12) patients were diagnosed as having an AMAN pattern: Almost all of these patients still had the AMAN pattern in weeks 3 to 4, whereas Patients 11 and 12 showed rapid increases in distal CMAP amplitude, though the value did not reach the normal range. Three patients (nos. 13, 14, and 21) showed F-wave absence as an isolated abnormality in the first studies, whereas two (nos. 13 and 14) developed AMAN several days later. The remaining patient (no. 21) experienced restoration of F waves associated with rapid clinical recovery, and nerve conduction studies were normal in week 4.

Five (20%) patients (nos. 16 to 20) showed the AIDP pattern in the initial studies. In all of them, the diagnosis of AIDP was based on prolonged distal latencies in two or more nerves. Two of these (nos.16 and 17) were rediagnosed as having AMAN 1 or 2 weeks later, because the distal latencies rapidly became normal. One patient (no. 18) showed rapid normalization of both the distal latencies and the CMAP amplitudes, and nerve conduction was near normal at week 3.

The electrodiagnostic findings in the anti-ganglioside-positive patients, therefore, were divided into three patterns: the AMAN pattern (simple reduction of CMAP amplitude), the AIDP pattern (prolonged distal latency), and the isolated absence of F waves with normal peripheral conduction. Figure 1 shows representative CMAP waveforms of the three patterns in the anti-ganglioside-positive patients. Sequential analysis showed that almost all of the anti-ganglioside antibody–positive patients eventually showed the AMAN pattern or normal/minor abnormalities.

To investigate the time course of electrodiagnostic abnormalities, we compared the sequential findings in the median distal latencies from weeks 1 through 6 in five anti-ganglioside-positive patients and -negative patients with the AIDP pattern in the first study (figure 2). Anti-ganglioside-positive patients showed normalization or mildly prolonged distal latencies for the next 5 weeks, in contrast to the anti-ganglioside-negative AIDP patients, who showed a progressive increase up to around week 5.

Figure 3 compares the sequential changes in the electrophysiologic parameters in the median nerve studies between the anti-ganglioside-positive and -negative groups. The mean distal latency, CMAP duration, motor nerve conduction velocity, and F-wave latency were distinct between the two patient groups, resulting from slowing of nerve conduction in the anti-ganglioside-negative group. There were progressive increases in the distal latency and F-wave latency up to month 2 in the anti-ganglioside-negative group.

*Sensory nerve conduction and electrodiagnosis.* The mean amplitude of the SNAP was clearly different

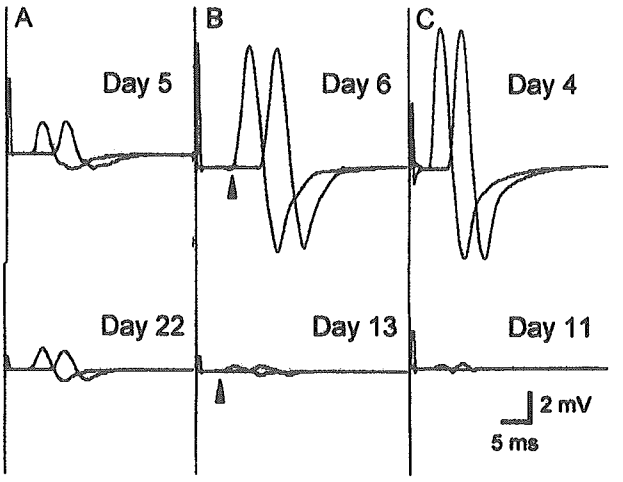


*Figure 1. Compound muscle action potentials (CMAPs) recorded from the abductor pollicis brevis muscle after median nerve stimulation at the wrist and elbow in patients with IgG anti-ganglioside antibodies (5 milliseconds, 2 mV/division). (A) Patient 4 showed simple reduction of the CMAP amplitudes (the acute motor axonal neuropathy [AMAN] pattern) on days 5 and 22 (see table E-1). (B) Patient 16 showed prolonged distal latency with normal CMAP on day 6. Note rapid shortening of distal latency and the decrease in distal CMAP amplitude on day 13. (C) Patient 13 showed normal CMAP amplitudes with the absence of F waves on day 4 and the AMAN pattern on day 11.*

throughout the course, reflecting pure motor involvement in most of the anti-ganglioside-positive group (see figure 3). Abnormalities of sensory nerve conduction were found for 3 of 25 (12%) of the anti-ganglioside-positive patients. These included only slightly decreased SNAP amplitudes, disproportional to markedly reduced CMAP amplitudes, and did not meet the criteria for AMSAN. On the other hand, 18 of 26 (69%) of the anti-ganglioside-negative patients showed sensory conduction abnormalities, and 14 of 18 showed markedly decreased or absent SNAPs in two or more nerves. All the 14 AIDP patients without anti-ganglioside antibodies showed sensory conduction abnormalities, and therefore there was no case of pure motor AIDP. Association of sensory nerve conduction abnormalities and final electrodiagnosis is summarized in table 3.

**Discussion.** Our results show that there are several patterns of early electrodiagnostic abnormalities in patients with anti-ganglioside antibodies. In the initial studies, 48% of patients showed the AMAN pattern, 20% the AIDP pattern, and 12% an isolated F-wave absence. Whereas the simple AMAN pattern was most frequently found as expected, some anti-ganglioside-positive patients showed distal nerve conduction slowing or the isolated absence of F waves. In sequential studies, the electrodiagnosis was rarely changed in patients initially diagnosed as having AMAN, but those with the AIDP pattern, or "F-wave absence," frequently showed different patterns of electrodiagnostic findings, and therefore their electrodiagnoses changed. Almost all of the

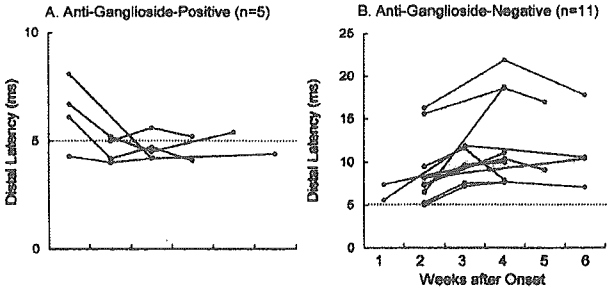


*Figure 2. Serial findings of distal latencies after median nerve stimulation at the wrist in the initial electrodiagnosis of acute inflammatory demyelinating polyneuropathy in individual patients with (A) and without (B) anti-ganglioside antibodies. Dotted line indicates the cut-off value of the criteria for demyelination.*

anti-ganglioside-positive patients eventually had AMAN or normal/minimal abnormalities in week 4. In the early phase of the disease, some anti-ganglioside-positive patients showed conduction slowing/block in the distal or proximal nerve segments but later developed axonal degeneration or showed rapid normalization of their conduction abnormalities. These findings suggest that patients with anti-ganglioside antibodies do not appear to have typical AIDP.

In contrast, most of anti-ganglioside-negative patients showed the AIDP pattern or minor abnormalities and rarely had their electrodiagnosis changed in the follow-up studies. Moreover, the time course of nerve conduction abnormalities was very similar to that of AIDP reported in previous studies.[7] Anti-ganglioside-negative patients were therefore likely to have typical AIDP. AIDP can be accompanied by secondary axonal degeneration, but in the anti-ganglioside-negative patients, transformation from the AIDP pattern to the AMAN pattern was not observed in this study.

All anti-ganglioside-positive patients with the AIDP pattern showed prolonged distal latencies, but the extent of abnormality was milder than in the anti-ganglioside-negative patients. Rapid resolution of the distal conduction slowing was found in some patients; persistent prolongation for weeks, without progressive increases, was observed in others. The time course was distinct from that of the anti-ganglioside-negative patients (see figure 2). The mechanisms of the transient or persistent prolongation of distal latency in the anti-ganglioside-positive patients are unclear. However, a number of factors other than demyelination can cause slowing of nerve conduction, such as loss of the fastest fibers, altered resting membrane potential (hyperpolarization or depolarization), and sodium channel inactivation.[23]

The blood–nerve barrier is anatomically deficient in the distal nerve terminals and nerve roots.[24] Therefore, anti-ganglioside antibodies would preferentially access those regions. The transient distal latency prolongation and F-wave absence found in

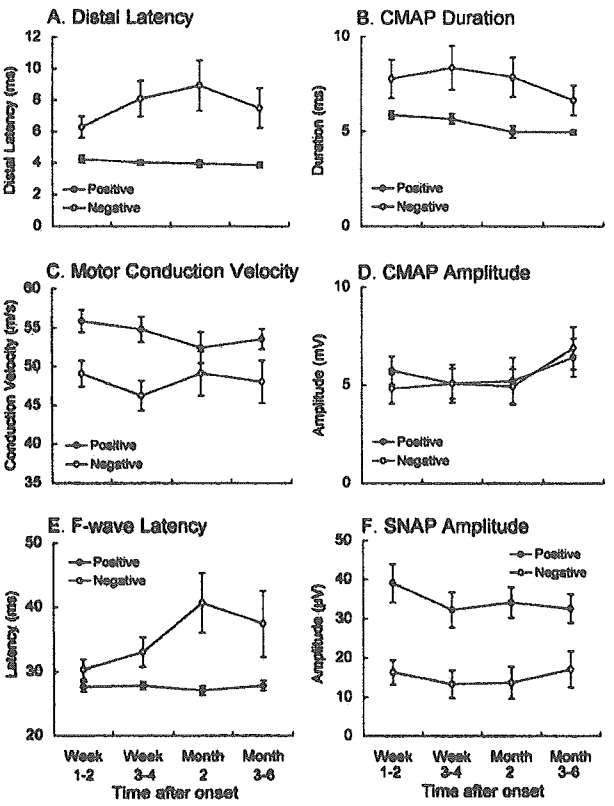


*Figure 3. Serial findings of motor distal latency (A), duration of compound muscle action potential (CMAP) (B), motor nerve conduction velocity (C), CMAP amplitudes (D), F-wave latency (E), and sensory nerve action potential (SNAP) amplitude (F) in median nerve conduction studies in IgG anti-ganglioside-positive (filled circles) and -negative (open circles) patients. The data are given as means ± SEM.*

our anti-ganglioside-positive patients may be explained by reversible conduction failure in the distal nerve terminals or nerve roots. Recent reports described patients with anti-ganglioside antibodies, who showed multifocal conduction block in the fore-

***Table 3*** *Sensory nerve conduction abnormalities and final electrodiagnosis in patients with Guillain–Barré patients*

| | IgG antibody to GM1, GM1b, GD1a, or GalNAc-GD1a | | | |
|---|---|---|---|---|
| | Positive, n = 25 | | Negative, n = 26 | |
| Sensory nerve conduction abnormalities | Yes, n = 3 | No, n = 22 | Yes, n = 18 | No, n = 8 |
| AIDP pattern | 1 | 1 | 14 | 0 |
| AMAN pattern | 1 | 16 | 0 | 3 |
| Others | 1 | 5 | 4 | 5 |

AIDP = acute inflammatory demyelinating polyneuropathy; AMAN = acute motor axonal neuropathy; Others = minor abnormalities (mild conduction abnormalities that did not meet the criteria for AIDP or AMAN) or normal.

arm segments of median or ulnar nerve,[25,26] but this type probably represents a rare variant and was not found in this study.

According to the electrodiagnostic criteria for AIDP and AMAN,[4] electrodiagnosis is based on motor nerve conduction studies "done during the first 2 weeks of illness," but our results showed that the electrodiagnosis of a considerable number of patients with anti-ganglioside antibodies changed during the first 4 weeks, and even within the first 2 weeks. In this regard, the electrophysiologic classification of GBS would be better determined based on sequential findings rather than only on the results of an initial study, and sequential findings up to weeks 3 to 6 appear to be important to make the final electrodiagnosis.

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# Antecedent infections in Fisher syndrome

## A common pathogenesis of molecular mimicry

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**Abstract**—*Objective:* To assess the production mechanism of anti-GQ1b autoantibody in Fisher syndrome (FS). *Methods:* The authors conducted a prospective case-control serologic study of five antecedent infections (*Campylobacter jejuni*, cytomegalovirus, Epstein–Barr virus, *Mycoplasma pneumoniae*, and *Haemophilus influenzae*) in 73 patients with FS and 73 sex- and age-matched hospital controls (HCs). Serologic evidence in FS patients of *C. jejuni* (21%) and *H. influenzae* (8%) infections was present significantly more often than in the HCs. None of the five pathogens examined was found in the 49 (67%) patients with FS. Anti-GQ1b IgG antibody was detected in most FS patients infected with *C. jejuni* or *H. influenzae*. Mass spectrometry analysis identified a *C. jejuni* strain (CF93-6) carrying a GT1a-like lipo-oligosaccharide (LOS) that had been isolated from an FS patient. Immunization of complex ganglioside-lacking knockout mice with the GT1a-like LOS generated IgG class monoclonal antibodies (mAbs) that reacted with GQ1b and GT1a. Thin-layer chromatography with immunostaining showed that anti-GQ1b mAb bound to the *C. jejuni* LOS (50% of the 20 FS-related strains) more commonly than in the Guillain–Barré syndrome (GBS)–related (7% of 70) or enteritis-related (20% of 65) strains. Anti-GM1 and anti-GD1a mAbs also reacted with the LOS from some FS-related strains (both 20%), but binding frequencies were higher in the GBS-related strains (74 and 57%). The GQ1b epitope was detected in 4 (40%) of the 10 FS-related *H. influenzae* strains but was absent in strains from patients with GBS ($n = 4$) and uncomplicated respiratory infections ($n = 10$). *Conclusions: C. jejuni* and *H. influenzae* are related to Fisher syndrome (FS) development, and production of anti-GQ1b autoantibody is mediated by the GQ1b-mimicking lipo-oligosaccharides on those bacteria. The causative agents remain unclear in the majority of patients with FS.

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Fisher syndrome (FS) is characterized by the acute onset of ophthalmoplegia, ataxia, and areflexia.[1] Its pathogenesis has been actively investigated,[2] and most patients with FS are found to have serum anti-GQ1b IgG autoantibodies during the acute phase of the illness that are cross-reactive with GT1a.[3-6] FS occurs subsequent to a wide variety of infections, most of which have been described in case reports, but there has been no case-control study of the antecedent infections. Previously, we reported positive serology for recent *Campylobacter jejuni* infection of 18% of 65 FS patients,[7] but no other Guillain–Barré syndrome (GBS)–related agent in FS has been investigated in a large number of patients. We also reported that in 7% of 70 FS retrospective cases, there was serologic evidence of recent *Haemophilus influenzae* infection.[8] A case-control study is needed to confirm its association with FS because the incidence of this infection is relatively rare.

*C. jejuni* strains isolated from FS patients had lipo-oligosaccharides (LOSs) that bore a terminal trisaccharide epitope mimicking GQ1b, GT1a, or GD3.[9-11] Immunization of mice with GT1a- or GD3-like LOS produced a monoclonal antibody (mAb) reactive against GQ1b and GT1a.[12] This raised the possibility that anti-GQ1b IgG antibody production is mediated by the trisaccharide epitope on bacterial LOSs. To verify this, it is necessary to prove that in a large number of isolates, FS is related to *C. jejuni* strains bearing a GQ1b-, GT1a-, or GD3-like LOS. We also reported that an *H. influenzae* type b serostrain had a GT1a-like LOS and hypothesized that ganglioside mimicry is involved in the development of FS after *H. influenzae* infection as well,[8] but whether such epitope is present in FS-related strains has yet to be determined.

Additional material related to this article can be found on the *Neurology* Web site. Go to www.neurology.org and scroll down the Table of Contents for the May 10 issue to find the title link for this article.

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We conducted a prospective case-control study of antecedent infectious serology in FS and then chemically determined the terminal oligosaccharide structure of the LOS on isolates obtained from an FS patient. By immunizing complex ganglioside-lacking knockout mice with the bacterial LOS carrying a GT1a epitope, we could clone mAb with reactivities against GQ1b and GT1a for use in examining whether the GQ1b-like LOS on the isolate is associated with FS.

**Methods.** *Patients.* We receive many requests from physicians throughout Japan to test for serum antiganglioside antibodies in patients with various neurologic disorders. On receipt of serum samples from FS patients, we have requested the primary physicians to send hospital control (HC) serum from sex- and age-matched (±5 years) patients without an autoimmune disease who did not have a history of recent enteritis but were in hospital at that time. If no patients fulfilled these criteria, we accepted as HCs sex- and age-matched (±5 years) healthy persons working at the hospital. We also collected sera from family members if possible. Between February 2000 and November 2003, we received 367 serum samples from FS patients, of which 73 paired samples from FS patients (men/women, 45/28; median age 32) and HC subjects (45/28; age 33) were available. Twenty control (8/12; age 49) samples from 18 families also were available. During the same period, we received 1,814 serum samples from patients with GBS, of which 73 (men/women, 45/28; median age 49) were selected randomly to be the disease controls. FS and GBS diagnoses were based on published clinical criteria.[13,14] Diagnosis of FS also was made for patients who initially presented with ophthalmoplegia, ataxia, and areflexia and then developed generalized muscle weakness. Although most had not been our patients, we collected their cases prospectively and asked the physicians whether diagnostic criteria had been fulfilled. One of the authors reviewed the medical records to verify the diagnoses.

*Infectious serology.* Recent *C. jejuni* infection was detected by an ELISA, as reported previously,[7] with altered criteria for seropositivity to increase its sensitivity and specificity (see appendix E-1 on the *Neurology* Web site at www.neurology.org). Under this condition, 43 of 47 GBS patients from whom *C. jejuni* had been isolated were judged positive within 4 weeks of GBS onset, whereas only 2 of 73 HC subjects with no history of recent enteritis were. Our assay therefore had a sensitivity of 91%, a specificity of 97%, and an efficiency of 95%. Evidence of recent *H. influenzae* infection was assayed serologically as reported elsewhere.[8] Infections by cytomegalovirus (CMV), Epstein–Barr virus (EBV), and *Mycoplasma pneumoniae* were also tested because a case-control study showed that they are related to the GBS development.[15] Serum IgM anti-CMV antibody and IgM anti-EBV capsid antigen antibody were tested using commercially available ELISA kits, Cytomegalo IgM(II)-EIA "SEIKEN" (Denka Seiken, Tokyo, Japan), and ETI-EBV-M reverse (DiaSorin, Stillwater, MN), according to the manufacturers' instructions. Serum anti-*M. pneumoniae* antibody was detected by the particle agglutination test (Serodia-Mycon II test kit; Fujirebio, Tokyo, Japan) after heating sera to 56 °C to inactivate complement.

*Antiganglioside antibodies.* We measured serum anti-GQ1b IgG/IgM/IgA and IgG antibodies against GT1a, GM1, and GD1a by ELISAs as described elsewhere.[7,16] Serum was considered positive when the optical density was ≥0.1 at a dilution of 1:500 for the IgG and 1:100 for the IgM and IgA antibodies. The IgG subclasses of anti-GQ1b antibody were examined in an ELISA with peroxidase-conjugated mouse anti-human γ1-, γ2-, γ3-, and γ4-chain-specific mAbs (Southern Biotechnology Associates, Birmingham, AL) as the secondary antibodies, as reported elsewhere.[17]

*Analysis of O-deacylated LOSs.* A *C. jejuni* strain, CF93-6, isolated from a patient with FS,[10] was used. Overnight growth of the strain on an agar plate was done as described previously,[18] except that we used 60 μg/mL proteinase K, 200 μg/mL RNAse A, and 100 μg/mL DNAse I. The *O*-deacylated LOS sample was analyzed by capillary electrophoresis–electrospray ionization–mass spectrometry (CE-ESI-MS), as described elsewhere.[19]

*Generation of anti-GQ1b mAbs.* Mice lacking the functional gene for β-1,4-*N*-acetylgalactosaminyltransferase (GM2/GD2 synthase; EC 2.4.1.92) were raised and their genotypes determined as described elsewhere.[20] They expressed no complex gangliosides, including neither GQ1b nor GT1a and therefore are an immune naive host and show strong IgG response to ganglioside-like LOS, whereas wild mice do not.[21] LOS was extracted from two *C. jejuni* strains (CF93-6 and CF90-26 [a GM1 epitope-bearing isolate from a GBS patient][22]) by the hot phenol-water technique,[23] after which the aqueous layer was dialyzed and centrifuged at 105,000 *g* for 16 hours. The mice were immunized intraperitoneally four times at 2-week intervals with 100 μg of LOS or 10 mg of a heat-killed lysate of *C. jejuni* dissolved in 50 μL of 2 mg/mL keyhole lympet hemocyanin solution (Sigma, St Louis, MO) that had been mixed with 50 μL of complete Freund adjuvant. Three days after final immunization with 50 μg of LOS in 50 μL of phosphate-buffered saline, mAbs were obtained as described elsewhere.[24] This research was approved by the Animal Care and Use Committee, Dokkyo University School of Medicine (approval no. 00-22). Mice were treated according to the Guidelines for the Care and Use of Laboratory Animals, Dokkyo University School of Medicine.

*Detection of ganglioside-like LOSs.* The presence of the GQ1b epitope was examined in *C. jejuni* and *H. influenzae* strains isolated from patients with FS (20 *C. jejuni* and 10 *H. influenzae*), GBS (70 *C. jejuni* and 4 *H. influenzae*), uncomplicated enteritis (65 *C. jejuni*), or a respiratory infection (10 *H. influenzae*). Anti-GQ1b IgG antibody was positive in patients from whom *C. jejuni* (FS, 18/20 [90%]; GBS, 3/70[4%]) or *H. influenzae* (FS, 10/10 [100%]; GBS, 0/4 [0%]) had been isolated. All the *H. influenzae* strains used were nontypable. Most of FS/GBS-related *C. jejuni* strains were isolated by one of the authors,[25] and all *H. influenzae* strains were obtained from hospitals throughout Japan. Ganglioside epitopes (GQ1b, GM1, and GD1a) were examined by thin-layer chromatography with immunostaining (*C. jejuni*) and ELISA (*H. influenzae*), as shown in appendix E-2.

*Statistical analysis.* Differences in the infectious serology frequencies of FS and HC were tested with the McNemar test, and frequency differences between groups were compared by the Fisher exact test. Differences in medians were examined by the Mann–Whitney *U* test. All calculations were done with SPSS 12.0J software (SPSS, Chicago, IL). A difference was considered significant when the two-sided $p$ value was $<0.05$.

**Results.** *Infectious serology.* Recent infectious agents were identified in 24 (33%) of the patients with FS, serologic evidence of recent *C. jejuni* (21%) and *H. influenzae* (8%) infections being more common than in the HCs, whereas frequencies of the other agents did not differ (table 1). As compared with the patients with GBS, the frequency of antecedent *C. jejuni* infection was lower and that of *H. influenzae* infection higher in patients with FS, but the differences were not significant. One family member, the 80-year-old husband of a *C. jejuni*-negative patient with FS, who had no history of recent infectious symptoms, was seropositive for *C. jejuni*. No family members were positive for *H. influenzae*. Positive serology for CMV infection was found for 3 of 20 family members, of whom 2 (mother and daughter) had coughs and nasal discharges at the time of sampling. Positive serology for more than one infection was found for only three (4%) of the FS patients: *C. jejuni* and *H. influenzae*, *C. jejuni* and CMV, and *C. jejuni* and EBV.

C. jejuni*-related FS.* Men predominated in the FS patients with *C. jejuni* infection (men/women, 11/4) as in patients without this infection (34/24). Teenagers and young adults (age <30) were proportionally higher in patients with *C. jejuni*-related FS (53%) than in the other patient groups (24%) ($p = 0.06$), but the median age did not differ significantly (28 vs 37 years old; $p = 0.19$). Patients with *C. jejuni* infection more often had a history of antecedent gastrointestinal symptoms (60 vs 35%; $p =$

***Table 1*** *Infectious serology*

| | FS | HC | Family control | GBS | Two-tailed $p$ value | | |
|---|---|---|---|---|---|---|---|
| | | | | | FS vs HC | FS vs family | FS vs GBS |
| n | 73 | 73 | 20 | 73 | | | |
| *Campylobacter jejuni* | 15 (21) | 2 (3) | 1 (5) | 23 (32) | <0.001* | NS | NS |
| *Haemophilus influenzae* | 6 (8) | 0 | 0 | 2 (3) | 0.04† | NS | NS |
| *Mycoplasma pneumoniae* | 3 (4) | 4 (5) | 1 (5) | 4 (5) | NS | NS | NS |
| Cytomegalovirus | 2 (3) | 0 | 3 (15) | 2 (3) | NS | NS | NS |
| Epstein–Barr virus | 1 (1) | 4 (5) | 0 | 1 (1) | NS | NS | NS |

Values in parentheses are percentages.

* Odds ratio, 9.1; 95% CI, 2.5–34.0.
† Odds ratio, 14.2; 95% CI, 1.1–15.8.

FS = Fisher syndrome; HC = hospital control; GBS = Guillain–Barré syndrome; NS = not significant ($p > 0.05$).

0.03). The neurologic features of facial palsy (27%), bulbar palsy (27%), limb weakness (13%), sensory disturbance (33%), and autonomic disturbance (7%) did not differ markedly among the groups. Anti-GQ1b and anti-GT1a IgG antibodies were present in all the patients with *C. jejuni* infection, more frequently than in the other patients ($p$ = 0.06 and 0.02), and anti-GM1 and anti-GD1a IgG antibodies also were detected more often ($p$ = 0.01 and 0.11) (figure 1). The anti-GQ1b antibody IgG subclass distribution was similar to that for patients without this infection (table 2). IgA and IgM anti-GQ1b antibodies were more frequent in patients with *C. jejuni* infection (80 and 73%) than in those without it (55 and 51%), but the differences did not reach significance ($p$ = 0.14 and 0.16).

H. influenzae*-related FS.* The median age of the six patients with *H. influenzae*-related FS was 54 years (range 14 to 86 years), and four were women. Upper respiratory tract infection preceded FS onset in four (67%) and gastrointestinal symptoms in one (17%). Bulbar palsy tended to be more frequent in patients with *H. influenzae* infection (50%) than in those without it (18%) ($p$ = 0.10), but the other neurologic features such as facial palsy (33%), limb weakness (17%), sensory disturbance (50%), and autonomic disturbance (0%) did not differ markedly. Five (83%) patients had anti-GQ1b and anti-GT1a IgG antibodies, but none had the anti-GM1 IgG antibody (see figure 1). The anti-GQ1b antibody IgG subclass distribution was similar to that found for the other patients (see table 2). A higher percentage of patients with *H. influenzae* infection had the IgM class of anti-GQ1b antibody (83 vs 54%; $p$ = 0.22), but IgA antibody frequency did not differ between the two groups (67 vs 60%; $p$ = 1.0).

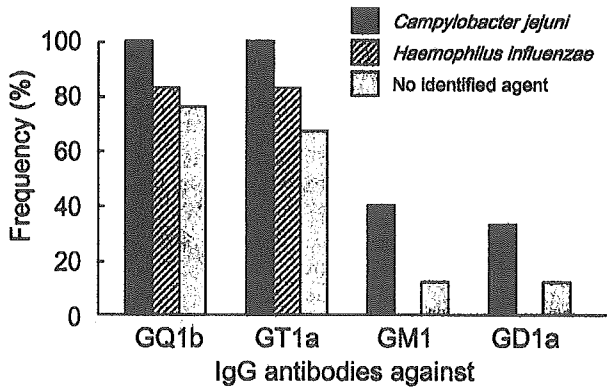


*Figure 1. Frequency of positive antiganglioside IgG antibody in patients with Fisher syndrome. Black columns = Fisher syndrome after* Campylobacter jejuni *infection (n = 15); hatched columns = Fisher syndrome after* Haemophilus influenzae *infection (n = 6); white columns = Fisher syndrome without identified agents (n = 49).*

*FS with no identified infection.* None of the five infections examined was found in 49 of the FS patients. Their median age was 35 years (range 4 to 81 years), and 30 were men. An antecedent upper respiratory tract infection was more frequent in this (88%) than the other (63%) group ($p$ = 0.03), whereas gastrointestinal symptom frequency did not differ (29 vs 42%; $p$ = 0.30). The neurologic features of facial palsy (16%), bulbar palsy (18%), limb weakness (16%), sensory disturbance (49%), and autonomic disturbance (8%) did not differ markedly between the groups. Antiganglioside IgG antibodies were less common in patients without identified infectious agents than in those with them (anti-GQ1b, $p$ = 0.12; anti-GT1a, $p$ = 0.04; anti-GM1, $p$ = 0.19; anti-GD1a, $p$ = 0.19) (see figure 1). The anti-GQ1b antibody IgG subclass distribution was similar to that found for the other FS patients (see table 2). Patients in this group less commonly had IgA (53 vs

***Table 2*** *IgG subclass classification of anti-GQ1b antibodies in patients with Fisher syndrome*

| | Fisher syndrome | | |
|---|---|---|---|
| | *C. jejuni* related | *H. influenzae* related | No identified agents |
| n | 15 | 5 | 37 |
| IgG subclass | | | |
| IgG1 | 14 (93) | 5 (100) | 35 (95) |
| IgG2 | 0 | 0 | 1 (3) |
| IgG3 | 7 (47) | 3 (60) | 22 (59) |
| IgG4 | 0 | 0 | 0 |

Values in parentheses are percentages.

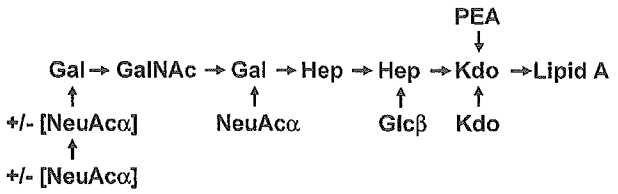


*Figure 2. Proposed lipo-oligosaccharide (LOS) outer core structures based on capillary electrophoresis–electrospray ionization–mass spectrometry analysis of an O-deacylated LOS sample from* Campylobacter jejuni *CF93-6. Gal = galactose; NeuAc = N-acetylneuraminic acid; GalNAc = N-acetylgalactosamine; Hep = L-glycero-D-manno-heptose; Kdo = 3-deoxy-D-manno-2-octulosonic acid; PEA = phosphoethanolamine; Glc = glucose.*

75%; $p = 0.08$) and IgM (49 vs 71%; $p = 0.09$), and anti-GQ1b antibodies were less common than in patients with identified infections.

*LOS structures of* C. jejuni *CF93-6.* Because an LOS sample from *C. jejuni* CF93-6 was used to immunize mice to obtain mAbs against specific gangliosides (see Methods and Results), we performed various analyses to better define the ganglioside mimics present in the outer cores of that sample. CE-ESI-MS analysis of the *O*-deacylated LOS sample from *C. jejuni* CF93-6 yielded various masses, in which the major species was $[M-4H]^{4-}$ = (3,937 Da). Other variants, shown in table E-1, are due to lipid A variations as well as to the presence or absence of the terminal sialic acid. The presence of one terminal sialic acid produces a GD1a mimic, whereas the presence of two yields a GT1a mimic (figure 2). The presence of two sialic acids as a chain (diNeu5Ac) in some of the variants was confirmed by CE-MS-MS and looking for the precursor ion at 581 Da (data not shown).

*mAb characteristics.* A clone (FS1) with anti-GQ1b IgG activity (IgG2bκ) was obtained from a mouse inoculated with *C. jejuni* (CF93-6) LOS, and two clones (FS2 and FS3, IgG2bκ) were obtained from mice inoculated with the *C. jejuni* heat-killed lysate. An ELISA showed that FS1 reacted with GD3 as well as with GQ1b and GT1a and that FS2 and FS3 specifically reacted with GQ1b and GT1a (table 3). Two clones were obtained from a mouse inoculated with *C. jejuni* (CF90-26) LOS. One (GB2), as reported elsewhere,[24] reacted strongly with GM1 and weakly with GD1a. The other (GB1) reacted strongly with GD1a but did not react with GM1.

*Ganglioside-like LOS.* FS1 was used to detect the GQ1b epitope on *C. jejuni* LOS because FS2 and FS3 had not been cloned at that time. Although we used two reagents (mAb and the patient's serum) in the detection of the ganglioside epitope, overall results were the same, except for some differences that probably were due to reagent sensitivities (table 4). The GQ1b epitope was more frequent in the FS-related *C. jejuni* strains than the GBS- and enteritis-related strains. In contrast, GM1 and GD1a epitope frequencies did not differ between the FS and enteritis strains. GBS-related strains more often had GM1- and GD1a-like LOSs than did the FS and enteritis ones.

Ganglioside epitopes on the *H. influenzae* LOS were examined quantitatively in an ELISA with FS3, GB2, and GB1 mAbs. The cut-off value for the presence of a ganglioside epitope was defined as an optical density of 0.1 based on results found for strains from patients with uncomplicated upper respiratory tract infections. Four (40%) of the 10 FS strains had a GQ1b-like LOS, whereas none of the GBS and uncomplicated strains did (figure 3). Of the four strains bearing a GQ1b epitope, three had neither the GM1 nor the GD1a epitope and one had the GM1 epitope. GM1 reactivities were present in the three (30%) FS-related strains, one of which also had GD1a reactivity. Unexpectedly, none of the four GBS-related strains was judged to have GM1 and GD1a epitopes.

*Table 3 Reactivity of monoclonal antibodies*

| Clone | Antiganglioside reactivity | | |
|---|---|---|---|
| FS1 | GQ1b (++) | GD3 (++) | GT1a (+) |
| FS2 | GQ1b (+++) | GT1a (++) | |
| FS3 | GQ1b (+++) | GT1a (+++) | |
| GB1 | GD1a (+++) | GalNAc-GD1a (+) | GT1b (+) |
| GB2 | GM1 (+++) | GD1a (+) | |

All clones were obtained by inoculating the mice lacking the complex gangliosides with *C. jejuni* (CF93-6; GT1a/GD1a/GM1 mimics) lipo-oligosaccharide (LOS) (FS1) or heat-killed lysate (FS2 and FS3) or *C. jejuni* (CF90-26; GM1/GD1a mimics) LOS (GB1 and GB2). IgG antibody activities against GM1, GM1b, GM2, GD1a, GalNAc-GD1a, GD1b, GD2, GD3, GT1a, GT1b, and GQ1b were tested in ELISAs. Results are expressed as relative activity at the appropriate dilution rate for each reagent. Only gangliosides bound (optical density [OD] of >0.5) by the reagents are shown. (+++) = OD of >2.5; (++) = OD 1.5–2.5; (+) = OD 0.5–1.5.

**Discussion.** Our study showed that *C. jejuni* and *H. influenzae* are causal agents of FS. No antecedent pathogens were identified in 67% of the FS patients studied, and no difference in the frequencies of CMV, EBV, and *M. pneumoniae* infections was found between FS and HC, whereas all were associated with GBS.[15] Although we changed the definition of serologic evidence of antecedent *C. jejuni* infection, its frequency in FS was similar to that reported earlier (18%).[7] It is noteworthy that most of the FS patients without identified infections had histories of antecedent upper respiratory infectious symptoms, indicative that respiratory infectious pathogens should be investigated as causal agents of FS. However, our results might merely reflect the low sensitivity of the infectious serology assays used. In spite of numerous infections reported as preceding FS onset,[26] there are only a few clues as to the major antecedent agents. β-Hemolytic streptococcal infection is an attractive candidate[27] because it may be followed by acute rheumatic fever and acute glomerulonephritis.[28] One serologic study, however, has reported that this antecedent infection is not common in FS.[29]

We showed that FS-related *C. jejuni* strains had a GQ1b/GT1a-like LOS more often than GBS- and enteritis-related ones, confirmation of the results of a small study showing that all four FS-related *C. jejuni* strains had a GQ1b-like LOS.[30] Moreover, for the first time, we determined that anti-GQ1b/GT1a mAb binds to LOSs from some FS-related *H. influen-*

*Table 4 Frequency of ganglioside epitopes on* C. jejuni *lipo-oligosaccharide*

| | | | | Two-tailed $p$ value | |
|---|---|---|---|---|---|
| | FS | GBS | Enteritis | FS vs GBS | FS vs enteritis |
| n | 20 | 70 | 65 | | |
| Resorcinol* reactive | 16 (80) | 66 (94) | 47 (72) | NS | NS |
| GQ1b epitope | | | | | |
| FS1† | 10 (50) | 5 (7) | 13 (20) | <0.001 | 0.02 |
| S7577 | 14 (70) | 11 (16) | 17 (26) | <0.001 | 0.001 |
| GM1 epitope | | | | | |
| GB2 | 4 (20) | 52 (74) | 25 (38) | <0.001 | NS |
| S6960‡ | 4 (20) | 49 (70) | 22 (34) | <0.001 | NS |
| GD1a epitope | | | | | |
| GB1 | 4 (20) | 40 (57) | 13 (20) | 0.005 | NS |
| S5174§ | 2 (10) | 42 (60) | 12 (18) | <0.001 | NS |

Values in parentheses are percentages.

* Reagent for staining sialic acid.
† Anti-GQ1b IgG-positive serum from a patient with FS.
‡§ Anti-GM1 ‡ and anti-GD1a § IgG-positive sera from patients with GBS.

NS = not significant ($p > 0.05$); FS = Fisher syndrome; GBS = Guillain–Barré syndrome.

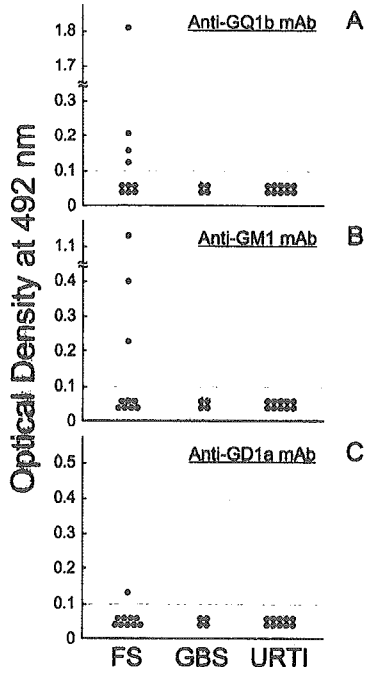


*Figure 3. Ganglioside epitopes on the* Haemophilus influenzae *lipo-oligosaccharide. Plots of individual antibody activities against ganglioside-like lipo-oligosaccharides on* Haemophilus influenzae *strains isolated from patients with Fisher syndrome (FS; n = 10), Guillain–Barré syndrome (GBS; n = 4), and uncomplicated upper respiratory tract infections (URTI; n = 10). Anti-GQ1b (FS3) (A), anti-GM1 (GB2) (B), and anti-GD1a (GB1) (C) were the monoclonal antibodies (mAbs).*

*zae* strains. These findings indicate a common pathogenesis of molecular mimicry in the development of *C. jejuni* and *H. influenzae*-related FS. The IgG subclass of anti-GQ1b antibody was almost always IgG1, IgG3, or both, as reported elsewhere,[31] IgG1 predominating irrespective of the antecedent infection identified. IgG2 was reported to be the main subclass of anti-GQ1b antibody in FS patients with an antecedent gastrointestinal infection (*C. jejuni* had been isolated from three of five patients),[32] whereas other investigators reported IgG1 and IgG3 in an FS patient from whom *C. jejuni* had been isolated.[33] Our and the other findings[32] apparently conflict. The reason is not clear. Our results agree with those of other studies as to the frequent presence of anti-GM1 IgG1 antibody in GBS cases in which there was *C. jejuni* serology.[17,33,34] We therefore believe that the IgG2 subclass of anti-GQ1b antibody is not associated with FS that develops subsequent to *C. jejuni* infection. Because the IgG subclass of antibody response is related closely to the types of antigens targeted and to T-cell dependency,[35] the similar IgG subclassifications of the anti-GQ1b antibody present in FS after *C. jejuni*, *H. influenzae*, or unidentified infections suggest that the mechanism of autoantibody production, probably mediated by ganglioside-like molecules on the infectious agent, is common in FS populations other than *C. jejuni*- and *H. influenzae*-related ones.

Ganglioside epitopes were not detected in some *C. jejuni* strains isolated from FS patients, but this does not indicate that other mechanisms than the molecular mimicry principle are applied to the antiganglioside antibody production in these patients. Ganglioside-like structure on *C. jejuni* LOS would