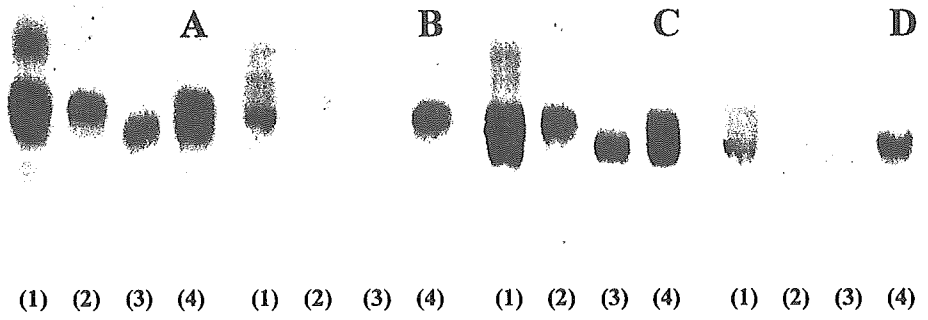

Fig 2. Thin-layer chromatogram (TLC) immunostaining of sera from Patients 1 and 3. TLC results in plates A and C are made visible by the orcinol reagent. Bovine brain gangliosides extracted by 0.1M ammonium acetate are in lane (1), GD1a (3μg) in lane (2), GD1b (3μg) in lane (3), and both GD1a and GD1b (3μg each) in lane (4). Plate B shows the TLC immunostaining of the serum from Patient 1, and plate D that of the serum from Patient 3. Compared with the slight immunostaining present in lanes (2) and (3), the overlapping portion of GD1a and GD1b in lane 4 is strongly immunostained.

The anti–GD1a/GD1b IgG antibody–positive patients also had antibody activities against a mixture of other gangliosides as shown in the Table.

Discussion

We first found an unidentified immunoreactive band in the position just below GD1a on TLC of a crude ganglioside fraction obtained through DEAE Sephadex A-25 column by extraction with 0.1M ammonium acetate. That led to the discovery of the antibody specific for the GD1a/GD1b ganglioside complex. We therefore focused our attention on the antibody to GD1a/GD1b in this article. The anti–GD1a/GD1b antibody–positive sera also had antibody activities to two

Table. Clinical Features and Antiganglioside Antibodies of GBS Patients with IgG Anti–GD1a/GD1b Antibody

Patient No.	Age (yr)	Sex	Antecedent Infection	F-score at Peak	Involved Cranial Nerves	Sensory Signs[a]	Corrected OD[b]			Antibodies to Other Ganglioside Complexes	Other Antiganglioside Antibodies
							Anti-GD1a/GD1b	Anti-GD1a	Anti-GD1b		
1	77	F	GI	5	9, 10	—[c]	1.24	0.15	0.23	GD1a/GM1, GM1/GT1b GD1b/GT1b	IgG: GT1b
2[d]	31	M	R	4	6	(++)	0.87	(−)	(−)	GD1a/GM1, GM1/GT1b GD1b/GT1b	(−)
3	30	M	GI	2	(−)	(−)	1.14	0.4	0.31	GD1a/GM1, GM1/GT1b GD1b/GT1b	IgG: GT1b IgM: GM1
4	70	M	R	5	3, 4, 5, 6, 7, 9, 10, 11, 12	(++)	0.98	0.14	0.15	GD1a/GM1, GM1/GT1b GD1b/GT1b	IgG: GQ1b
5	59	M	GI	4	7, 9, 10, 11, 12	(−)	1.16	0.18	0.83	GD1a/GM1, GM1/GT1b	(−)
6	47	F	GI	4	3, 4, 6, 7, 9, 10	(+)	0.92	(−)	(−)	GD1a/GM1, GM1/GT1b GD1b/GT1b	(−)
7	29	F	R	5	7, 9, 10, 12	(+)	0.61	(−)	(−)	GD1a/GM1	IgG: GM1
8	52	F	GI	5	5, 8, 9, 10, 11	(−)	0.95	(−)	0.15	GD1a/GM1, GM1/GT1b GD1b/GT1b	IgG: GM1 IgM: GM1

[a]Criteria for sensory signs: (−), no sensory signs or symptoms; (+), only paresthesia or dysesthesia; (++), sensory deficits
[b]Corrected optical density (OD) values of anti-ganglioside antibodies: corrected by subtracting the OD of a control (uncoated) well. (−) = negative result. F-score at peak: patient disabilities were graded on the Hughes functional grading scale [14]; 0 = no symptoms, 1 = minor signs or symptoms, 2 = able to walk 5m without support but incapable of manual work, 3 = able to walk 5m only with a cane, appliance, or support, 4 = bed- or chair-bound, 5 = requiring assisted ventilation, 6 = dead.
[c]No available data.
[d]Patient A

GI = gastrointestinal infection; R = respiratory tract infection.

or more of ganglioside complexes as shown in the Table: GD1a/GM1, GD1b/GT1b, and GM1/GT1b. We plan to investigate intensively the presence of antibodies to the various ganglioside complexes using sera from a larger group of GBS patients.

In view of characteristics of glycosphingolipids forming clusters extensively in the plasma membrane,[7] it is no wonder that clustered glycoepitopes of ganglioside complexes in the membrane are targeted by serum antibodies in GBS patients. No studies, however, have ever found antibodies to the ganglioside complex in sera from patients with GBS and related disorders. OD values of the anti–GD1a/GD1b antibodies were much higher than those of the anti–GD1a or anti–GD1b antibodies, and anti–GD1a/GD1b antibody–positive sera often showed little or no reactivity to GD1a or GD1b in the ELISAs (see Table), indicative that mixing GD1a with GD1b produced a ganglioside complex and new glycoepitopes that differ from those of GD1a or GD1b alone. What the structure of these glycoepitopes formed in the GD1a and GD1b mixture has yet to be determined.

Glycosphingolipids penetrate the outer leaflet of the plasma membrane via ceramide and are preferentially packaged with cholesterol, forming lipid rafts. These lipid rafts, protein-linked microdomains in the plasma membrane, are called detergent-insoluble glycolipid-enriched complexes or glycosphingolipid-enriched membranes. Within the plasma membrane microdomains, glycosphingolipids, particularly gangliosides, are believed to interact with important transmembrane receptors or signal transducers involved in cell adhesion and signaling.[7,8] Because proteins in the microdomains are not free to spread over the plasma membrane, specific ones tend to be concentrated within microdomains that often are essential for protein function. Antibodies to a ganglioside complex therefore may alter the function of the axon or Schwann cell through their binding to clustered epitopes of glycosphingolipids in the plasma membrane microdomains. Consequently, they may directly induce nerve conduction failure and severe disability in patients with GBS.

Some antiganglioside antibodies are correlated with the clinical phenotypes of GBS[2]; an anti–GQ1b antibody is associated with Miller Fisher syndrome[9] and the anti–GM1, GD1a, and GalNAc-GD1a antibodies with pure motor type of GBS.[6,10–13] Anti–GD1a/GD1b antibody also may be correlated with a GBS phenotype. Anti–GD1a/GD1b antibody–positive patients with GBS tend to have severe disabilities and cranial nerve deficits. Clinical studies of larger numbers of patients with GBS are needed to clarify the clinical importance of anti–GD1a/GD1b antibody.

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Effects of phospholipids on antiganglioside antibody reactivity in GBS

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Abstract

Serum antibody activities to mixtures of a ganglioside and various phospholipids were compared with those to a ganglioside alone in 30 anti-GM1 IgG-positive GBS patients and 30 anti-GQ1b IgG-positive Miller Fisher syndrome (MFS) patients. Anti-GM1-positive sera had higher antibody reactivities against a mixture of GM1 and several phospholipids including PA, PI and PS, than against GM1 alone. In contrast, in case of anti-GQ1b antibody, no phospholipid provided significant enhancement. Sphingomyelin provided decrease of the activity for both anti-GM1 and anti-GQ1b IgG. The effects of phospholipids must be considered to determine the pathogenetic role of antiganglioside antibodies in GBS and MFS.
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Keywords: GBS; Antiganglioside antibody; Phospholipids

1. Introduction

Antiganglioside antibodies frequently are present in patients with Guillain-Barré syndrome (GBS) and its variant, Miller Fisher syndrome (MFS). Anti-GM1 IgG antibodies are associated in particular with the pure motor variant of GBS (Visser et al., 1995), or with acute motor axonal neuropathy (Hafer-Macko et al., 1996), whereas anti-GQ1b IgG antibodies are associated with MFS (Chiba et al., 1992). Antiganglioside antibodies are useful diagnostic markers as well as possible pathogenetic factors in GBS.

Recently, we reported that IgG antibody in acute GBS sera had a higher titer against a mixture of GM1 and phosphatidic acid (PA) than against GM1 alone (Kusunoki et al., 2003). The use of a mixture antigen of GM1 and a phospholipid may provide us with an improved method of the antibody assay for the diagnosis of GBS. To investigate which phospholipid is the most effective for the enhancement of the anti-GM1 IgG activities and whether such an enhancement is generally observed in the antiganglioside antibodies in GBS or its variant, we examined IgG reactivities against GM1 or GQ1b with and without various phospholipids.

2. Materials and methods

2.1. Serum samples

Sera were obtained from 30 GBS patients who had anti-GM1 IgG antibodies and from 30 MFS patients who had anti-GQ1b IgG antibodies.

2.2. Antibody assay

Antiganglioside antibody reactivities were measured by the enzyme-linked immunosorbent assay (ELISA), as described elsewhere (Kusunoki et al., 1994). Nine phospholipids were used: phosphatidic acid (PA), phosphatidylethanolamine (PE), phospatidylinositol (PI), phosphatidylserine (PS), lysophosphatidylethanolamine (LPE), lysophosphatidylcholine (LPC), phosphatidylcholine (PC), sphingomyelin

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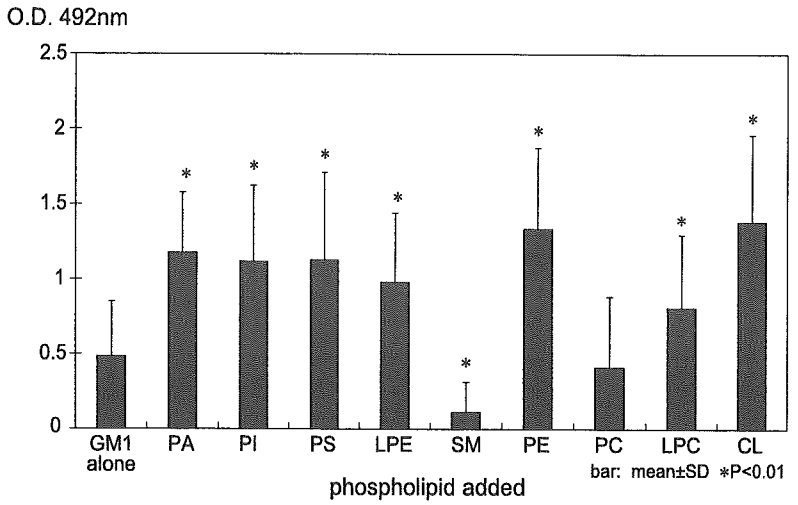

Fig. 1. Means of the antibody activities for 30 anti-GM1 IgG-positive GBS patients. Activity increased when a mixture of GM1 and PA, PI, PS, LPE, PE, LPC or CL was the antigen and decreased when a mixture of GM1 and SM was.

(SM) and cardiolipin (CL) (all purchased from DOOSAN Serdary Research Laboratories, USA).

Wells of 96-well microtiter plates were coated with 200 ng of ganglioside (GM1 or GQ1b), an antigen mixture of 100 ng each of ganglioside and phospholipid, or 200 ng of phospholipid only. An uncoated well was the control. Each OD value was corrected by subtraction of the control well OD. Serum with a corrected OD of more than 0.1 was considered positive. The antibody activity of each patient's serum was expressed as the mean of the corrected ODs of two independent assays.

2.3. Statistics

Antibody activities against a ganglioside and against a mixture of ganglioside and phospholipid were compared by the Wilcoxon signed-ranks test. Significance was considered positive if $p<0.01$.

3. Results

The binding activity of anti-GM1 IgG-positive GBS sera to a mixture of GM1 and a phospholipid was compared to that of GM1 alone. The mean antibody activity against a mixture of GM1 and phospholipid was significantly higher than against GM1 alone when the phospholipid was PA, PI, PS, LPE, PE, LPC or CL, whereas it was significantly lower when the antigen was a mixture of GM1 and SM (GM1/SM) (Fig. 1). Of the individual sera, more than 70% of the GBS patients had higher antibody activity against a mixture of GM1 and the phospholipid PA, PI, PS, LPE, PE, LPC or CL than against GM1 alone. In contrast, almost all had significantly lower antibody activity against GM1/SM than against GM1 alone. The antibody activity against the mixture of GM1 and SM remained decreased even when a mixture of 200 ng GM1 and 100 ng phospholipid per well was used instead of 100 ng each.

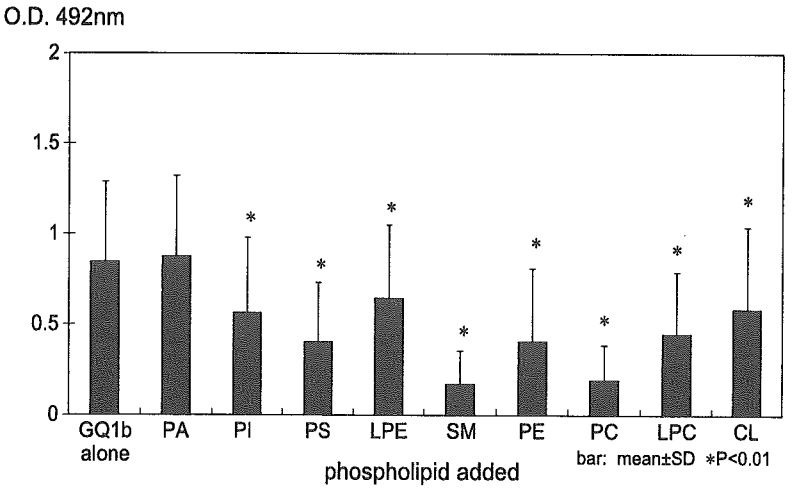

Fig. 2. Means of the antibody activities for 30 anti-GQ1b IgG-positive MFS patients. Means of the antibody activities increased only when a mixture of GQ1b and PA was the antigen. The other phospholipids produced no enhancement.

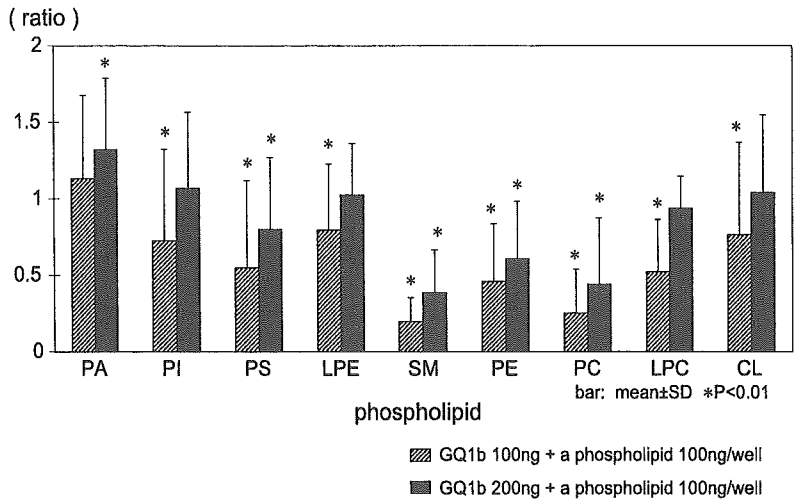

Fig. 3. Antibody reactivity against a mixture divided by that against GQ1b alone. Columns give the means of the ratios. Activities in MFS against mixtures (except GQ1b and PA) were lower than against GQ1b alone. When the mixture contained 200 ng of GQ1b and 100 ng of phospholipid per well, instead of 100 ng each, binding activity against the mixture was restored to a level similar to that against GQ1b alone (200 ng/well) for PI, LPE, LPC and CL.

Results for the anti-GQ1b IgG-positive sera clearly differed from those for anti-GM1 IgG-positive sera; only PA had a slight enhancing effect, but it did not reach statistical significance. The other phospholipids did not have an enhancing effect. Antibody activities against mixtures were lower than against GQ1b alone (Fig. 2).

When a mixture of 200 ng GQ1b and 100 ng phospholipid per well was used instead of 100 ng each, serum antibody binding activity against a mixture was restored to a level similar to that against GQ1b alone for the phospholipids PI, LPE, LPC and CL but not PS, SM, PE or PC (Fig. 3).

The control experiments showed that IgG antibodies against phospholipids were negative in most cases, indicative that the reaction of the IgG antibody with phospholipid did not account for the phospholipid enhancement of anti-GM1 IgG antibody activity described above. Some GBS patients had low antibody activity titers against LPE, PE, PC, LPC and CL. However, the antibody reactivities against mixture antigens (GM1/LPE, GM1/PE, GM1/PC, GM1/LPC and GM1/CL) were still much higher than those against GM1 alone even if ODs of phospholipid coated wells were subtracted from those of GM1/phospholipid wells.

Of the 30 patients with GBS, 4 had both anti-GM1 IgG and anti-GM1 IgM. We examined the reactivity of the IgM antibodies from those four patients with a mixture of GM1 and phospholipid. No enhancing effect like that obtained with IgG antibodies occurred with the IgM antibodies. When GM1 was mixed with SM, IgM activity decreased like that for IgG.

4. Discussion

In the previous study, maximal binding activities were observed at a GM1/PA ratio of 1:1 when varying ratios of GM1 and PA of mixture antigen were used (Kusunoki et al., 2003). In this study, we therefore investigated antibody activities against a mixture at a ganglioside/phospholipid ratio of 1:1. Anti-GM1 IgG-positive GBS sera had higher antibody activities against a mixture of GM1 and the phospholipid PI, PS, LPE, PE, LPC or CL, as well as PA, than against GM1 alone. In our preliminary study, some anti-GM1 IgG-negative patients had IgG reactivity against a mixture of GM1 and such phospholipids as PI, PS, CL as well as PA. A mixture antigen of GM1 and such a phospholipid as PA, which provided significant enhancement of the anti-GM1 IgG reactivity in GBS sera, may be more useful than GM1 alone in ELISA for the diagnostic test of GBS.

Effects of the addition of a phospholipid on the activities of anti-GQ1b IgG-positive sera differed. A phospholipid addition to GQ1b antigen did not produce a significant increase in antibody activity, and activity was decreased when a mixture of GQ1b and a phospholipid (except PA) was the antigen.

The cause of the difference in the anti-GM1 IgG and anti-GQ1b IgG reactivities remains to be determined. It is possible that the physiochemical differences of GM1 and GQ1b make the differences of the effects of phospholipids on binding activities of antibodies. However, anti-GM1 antibody activities are not necessarily enhanced by the addition of PA to the antigen, as seen in some GBS sera and in the rabbit antisera (Kusunoki et al., 2003). The most probable reason may be the difference in the preceding infection. The major phospholipid constituents in Gram-negative bacteria are PE, phosphatidylglycerol and CL. PS and PA are present as minor components (Huijbregts et al., 2000), but these bacteria have no SM (Zien et al., 2001; Carman and Henry, 1999). This indicates that anti-GM1 IgG-positive sera reactivity increased when a phospholipid present in *Campylobacter*

jejuni, a Gram-negative bacterium, was added to the antigen mixture. Respiratory rather than gastrointestinal infections, however, precede onset in most anti-GQ1b IgG-positive patients; those with Miller Fisher syndrome. The difference in the preceding infectious agent may, at least in part, explain why the reactivity of anti-GQ1b IgG-positive serum was not enhanced by the addition of those phospholipids that increased the reactivity of anti-GM1 IgG-positive serum.

Whether antibodies with high reactivity against a mixture of GM1 and phospholipid rather than against GM1 alone recognize a conformational epitope formed by GM1 and a phospholipid has yet to be clarified. The antibodies possibly have specificity for GM1 which undergoes conformational change in association with a phospholipid, as reported for β2-glycoprotein I and anticardiolipin antibodies (Matsuura et al., 1994). Phospholipids also may affect the binding force between antibodies and GM1. Because the phospholipids composition surrounding ganglioside antigen can influence antibody binding, susceptibility to injury can be contributed by membrane phospholipids content. The composition and distribution of each phospholipid at such possible target sites as the paranodal myelin, axolemma, and neuromuscular junction, needs to be clarified in future studies.

The effect of SM in decreasing the antibody activity of both anti-GM1 IgG-positive and anti-GQ1b IgG-positive sera seems to be of importance. Gangliosides are widely distributed throughout the nervous system, individual ones sometimes being dense at a particular site. For example, GQ1b is present in the spinal roots and in all the cranial nerves (Chiba et al., 1997), but is densely localized in the paranodal regions of oculomotor, trochlear and abducens nerves. Ophthalmoplegia may be due to the specific binding of anti-GQ1b antibodies to these regions (Chiba et al., 1993). The decrease seen in antiganglioside antibody reactivity in GBS and MFS caused by SM may be why antiganglioside antibody does not cause ubiquitous damage to the neurological system but damages only sites where gangliosides are densely localized.

Greater attention must be paid to the effects of the phospholipids that surround gangliosides on cell membranes of the human nervous system in order to determine the pathogenetic role of antiganglioside antibodies in autoimmune neuropathies. To better understand how phospholipids influence the interaction of antibodies and ganglioside in vivo, further investigation by the use of such system that mimic the myelin membrane as liposomes consisting predominantly of cholesterol, PE and PC is needed.

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RESEARCH REPORT

Harmful effects of anti-GalNAc-GD1a antibodies and TNF-α on rat dorsal root ganglia

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Abstract The clinical characteristics of five (22%) of 23 patients with Guillain-Barré syndrome (GBS), whose serum contained immunoglobulin G (IgG) antibodies to the ganglioside *N*-acetylgalactosaminyl GD1a (GalNAc-GD1a), included pure motor weakness of the axonal type. These patients had a relatively good prognosis, but displayed higher serum tumor necrosis factor-alpha (TNF-α) titers than the other GBS patients. We examined the effect of serum from these patients with IgG anti-GalNAc-GD1a antibodies on neurites from cultured rat dorsal root ganglia (DRG) and found it to damage the myelin in well-elongated DRG neurites and monolayer cultures of Schwann cells and neurons. In the regeneration model, serum from these patients delayed neurite extension and inhibited Schwann cell proliferation. Neurons in cultured monolayers showed vacuolation and decreased rapidly in number. Schwann cells were also vacuolated and readily detached from the substratum. The effects of IgG anti-GalNAc-GD1a antibodies purified from one of the patients, rabbit serum after immunization with GalNAc-GD1a, and recombinant TNF-α were also examined. IgG anti-GalNAc-GD1a antibodies mainly inhibited the regeneration and preservation of neurons, while TNF-α mainly induced morphological changes in well-proliferated Schwann cells and myelin.

Key words: axonal type Guillain-Barré syndrome, dorsal root ganglia, IgG anti-GalNAc-GD1a antibody, TNF-α

Introduction

Various antibodies against gangliosides detected in the serum of patients with Guillain-Barré syndrome (GBS) have been recognized as important causes of the disorder *(Willison and Yuki, 2002; van Sorge et al., 2004)*. Recently, ganglioside complexes were recognized as new target antigens in GBS *(Kaida et al., 2004)*. The clinical characteristics of GBS patients with serum immunoglobulin G (IgG) antibodies to the ganglioside *N*-acetylgalactosaminyl GD1a (GalNAc-GD1a), which included pure motor weakness of the axonal type, have been the focus of recent studies *(Kusunoki et al., 1994; Ang et al., 1999; Hao et al., 1999; Kaida et al., 2001)*, and we previously immunolocalized GalNAc-GD1a in human ventral roots, dorsal roots, intramuscular nerves, and sural nerves *(Kaida et al., 2003)*. The ability of IgG anti-GalNAc-GD1a antibodies to block neuromuscular transmission has recently been reported *(Taguchi et al., 2004)*. The pathogenetic significance of IgG anti-GalNAc-GD1a antibodies in axonal type GBS, however, has not yet

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been clarified. In contrast, the roles of a number of cytokines have been examined in various immune-mediated demyelinating diseases, including GBS *(Sivieri et al., 1997; Zhu et al., 1997; 1998)*. There is a striking correlation, for example, between serum tumor necrosis factor-α (TNF-α) titer and severe neuropathy in patients with GBS *(Sivieri et al., 1997)*.

We treated 23 GBS patients between 1996 and 2000, five (22%) of whom, with the axonal type GBS, had IgG anti-GalNAc-GD1a antibodies. Our observations have been reported previously *(Ang et al., 1999; Hao et al., 1999; Kaida et al., 2001)*. These five patients had significantly higher serum TNF-α titers than the other 18 GBS patients who had other types of anti-ganglioside antibodies, yet despite this had a relatively good prognosis. In the present study, we examined the effects of serum from these patients with axonal type GBS on neurites from cultured rat dorsal root ganglia (DRG). Using the same system, we also examined the effects of purified IgG anti-GalNAc-GD1a antibodies from one of the patients and recombinant TNF-α. The effects of these agents on monolayer cultures of DRG neurons and Schwann cells were subsequently investigated to determine the pathogenetic significance of anti-GalNAc-GD1a antibodies in axonal type GBS.

GBS involves motor weakness, although whether the DRG culture system can serve as a GBS model is not clear. We used it because GalNAc-GD1a can be detected in cultured DRG and roots by immunoblotting. Sensory nerve action potential was found to be decreased in some patients with IgG anti-GalNAc-GD1a antibodies who were asymptomatic, but whose posterior roots had histochemical immunoreactivity against GalNAc-GD1a, indicative of sensory root involvement *(Kaida et al., 2003)*.

Materials and Methods

Samples

Serum was collected from the five patients with high IgG anti-GalNAc-GD1a and TNF-α titers 10–14 days after the onset of neurological signs, and just before the start of treatment, and from one of them (patient 4) 3 weeks after the onset of illness which had not yet been treated. Two of these patients (patients 2 and 5) also had IgG anti-asialo GM1 (Table 1). Control serum was obtained from three healthy individuals (human serum, HS) and from patient 1 following recovery, 6 months after the onset of illness (Pt.1–6m), at which time there were almost no IgG anti-GalNAc-GD1a antibodies and the TNF-α titer was very low. Serum from GBS patients with other types of anti-ganglioside antibodies and high TNF-α titers (patients 6 and 7) was also collected. The clinical characteristics of these patients are summarized in Table 1. Serum was stored at −80°C, and half of each sample was inactivated at 56°C for 30 min before use. Because noninactivated HS had a supportive effect on cultures, inactivated HS (inact-HS) was used as control serum. We also examined the effects of purified anti-GalNAc-GD1a antibodies and recombinant human TNF-α (stock solution: 10 μg/mL; Genzyme) in the presence of inact-HS. IgG anti-GalNAc-GD1a antibodies were isolated from the serum of patient 1 by adsorption to GalNAc-GD1a (Calbiochem-Novabiochem) bound to octyl Sepharose CL-4B (Pharmacia). Adsorbed anti-GalNAc-GD1a antibodies were eluted with 3 M NaSCN and dialyzed against 0.01 M phosphate-buffered saline (pH 7.2). Serum that had passed through the octyl Sepharose column (adsorbed serum with GalNAc-GD1a) and inact-HS served as control sera for IgG-purified anti-GalNAc-GD1a antibodies and TNF-α.

Additionally, we examined the effects of rabbit serum with a high titer of IgG anti-GalNAc-GD1a antibodies *(Kaida et al., 2003)*.

Anti-ganglioside antibody and TNF-α titers

Anti-ganglioside titers were determined by an enzyme-linked immunosorbent assay (ELISA) at the Department of Neurology, University of Tokyo, as described previously *(Kusunoki et al., 1992)*. TNF-α titers were determined by ELISA, using a kit (Immunotech), according to the manufacturer's instructions.

Dorsal root ganglion dissection and culture

DRG were excised from 7-day-old Wistar rats under ethyl ether anesthesia, and cultures derived from them were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. Modified whole DRG cultures were established, as described elsewhere *(Peterson and Murray, 1965; Wood, 1976)*. Neuron and Schwann cell monolayer cultures were established as described by Fukuda *et al. (1991)*. Morphological observations were made using phase-contrast microscopy. Collagen-embedded DRG and neurites fixed with periodate-lysine-paraformaldehyde *(McLean and Nakane, 1974)* were stained by standard immunohistochemical procedures. The primary antibodies were a rabbit anti-neurofilament polyclonal antibody for the visualization of axons and mouse anti-myelin basic protein and anti-S100 protein monoclonal antibodies (1 : 300, Chemicon International) for the visualization of myelin. Fluorescence isothiocyanate-labeled anti-rabbit IgG (1 : 300, Jackson IRL) and Cy3 anti-mouse IgG (1 : 300, Jackson IRL) were used as secondary antibodies.

To examine the effects of GBS serum, samples were added to the medium at a concentration of 10%

Table 1. Clinical characteristics of Guillain-Barré syndrome patients.

Patient	Age (years)	Sex	*Campylobacter jejuni*	Antibody IgG	Clinical findings	Electrophysiological study
1	25	Female	Stool (+)	Anti-GalNAc-GD1a	Motor weakness	MCV: 42–60 m/s, CMAP: 0.04–0.08 mV, dispersion (+), SCV: 51–59 m/s, SNAP: 9–25 μV
2	29	Male	Serum (+)	Anti-GalNAc-GD1a, anti-asialo GM1	Motor weakness, dysesthesia	MCV: 40–57 m/s, CMAP: 0.15–1 mV, dispersion (+), SCV: 45–61 m/s, SNAP: 0.02–1 μV
3	9	Female	Serum (+)	Anti-GalNAc-GD1a	Motor weakness	MCV: 34–38 m/s, CMAP: 0.03–0.06 mV, dispersion (+), SCV: 57–66 m/s, SNAP: 28–31 μV
4	18	Male	Stool (+)	Anti-GalNAc-GD1a	Motor weakness	MCV: 55–63 m/s, CMAP: 2.3–2.6 mV, dispersion (–), SCV: 54–67 m/s, SNAP: 15–16 μV
5	50	Female	Serum (+)	Anti-GalNAc-GD1a, anti-asialo GM1	Motor weakness, cranial nerve VII, XI, X	MCV: 41–48 m/s, CMAP: 0.2–1.2 mV, dispersion (+), SCV: not done
6	35	Male	Stool (+)	Anti-GM1, anti-GD1b	Motor weakness, sensory disturbance	MCV: 44–57 m/s, CMAP: 0.29–2.4 mV, dispersion (+), SCV: 54–63 m/s, SNAP: 14–19 μV
7	17	Female	Not done	Anti-GM1, anti-GD1b	Motor weakness, sensory disturbance	MCV: 40–41 m/s, CMAP: 0.5–0.8 mV, dispersion (+), SCV: not done

SNAP, sensory nerve action potential. Complement-fixation tests for anti-*C. jejuni* antibody and stool cultures for *C. jejuni* were performed at the Department of Microbiology of the Metropolitan Tokyo Research Laboratory of Public Health.

(v/v), replacing fetal bovine serum (FBS). Seven or 14 days later, the DRG or monolayer cultures were examined. For studies with whole DRG embedded in collagen, four to six DRG were cultured with each serum sample, each experiment being performed in triplicate. For studies with monolayer cultures, investigations were performed in triplicate. Each experiment was performed in triplicate.

Effect of GBS serum on sufficiently extended neurites, preserved neurons, and proliferated Schwann cells in monolayer culture

We investigated the effects of GBS serum samples on neurites extending from DRG embedded in collagen (Nitta Gelatin), cultured every 2 days for 14 days in medium 1 or 2, as well as on preserved neurons and well-proliferated Schwann cells in monolayer cultures. DRG monolayers digested with 0.15% collagenase (Wako Pure Chemicals) were cultured in medium 3 for 7 days, and then for a further 7 days in medium 2, in which Schwann cells proliferated. We also examined the effect of each GBS serum on neurons after 4 days of treatment with 0.1% × 10^{-5} M cytosine-1-β-D-arabinofuranoside (Wako Pure Chemicals) in medium 3, under which conditions neurons were well preserved despite the absence of Schwann cells. The composition of each medium is given in Table 2.

Effect of GBS serum on neurite extension, Schwann-cell proliferation, and nerve preservation: regeneration model

Each GBS serum sample was added to the culture medium from the initiation of culture, and its effects on

Table 2. Composition of each medium.

Medium 1	78% modified Eagle's medium (MEM; Gibco BRL), 10% fetal bovine serum (FBS; Gibco BRL), 2% chick embryo extract (CEE; Gibco BRL), 10% 0.15 M KCl (Wako Pure Chemicals), 5 g/L glucose (Wako Pure Chemicals), 1% 7S-nerve growth factor (NGF; Chemicon International), 10^{-5} M cytosine-1-β-D-arabinofuranoside (Wako Pure Chemicals), 10^{-5} M 5-fluoro-5-deoxyuridine (Gibco BRL), 10^{-5} M uridine (Sigma); MEM contained 78% Eagle's minimum essential medium (Gibco BRL) supplemented with 2.2 g/L of $NaHCO_3$ (Wako Pure Chemicals), 5 g/L glucose, and 250,000 IU of penicillin (ICN Biomedicals)
Medium 2	65% MEM, 25% FBS, 10% CEE, and 0.1% NGF
Medium 3	Dulbecco's modification of Eagle's medium (50%) and Ham's medium (50%) (Gibco BRL), 15 mM sodium HEPES and L-glutamine (0.365 g/L; Sigma), supplemented with transferrin (100 mg/L; Sigma), putrescine (16 mg/L; Sigma), insulin (5 mg/L; ICN Biomedicals), progesterone (6.5 g/L; Sigma), sodium selenite (30 nM; Sigma), penicillin (0.2 mg/L), streptomycin (200 IU/L; ICN Biomedicals), sodium bicarbonate (1.0 g/L), and 7S-NGF (0.1% v/v of a 10 ng/mL solution; Chemicon)

DRG neurite elongation and dissociated neurons and Schwann cells investigated. After 1–2 weeks of culture of whole DRG, the 20 longest neurites extending from each were measured. Neurite extension was defined in terms of multiples of the short diameter of the parent ganglion. The effects of GBS serum samples on numbers of neurons and Schwann-cell proliferation in dissociated DRG monolayers were examined. The numbers of neurons in five fields per dish were counted at ×100 magnification. Comparisons were made by the Kruskal–Wallis test. Statistical significance was at $p < 0.05$.

GalNAc-GD1a in rat DRG and roots

The lipid fraction from rat 'DRG and roots' was subjected to thin-layer chromatography (TLC; Macherey-Nagel), the plates being developed with chloroform/methanol/0.2% $CaCl_2$ (50 : 45 : 10 v/v). Plates were immunostained with rabbit anti-GalNAc-GD1a antibodies *(Kaida et al., 2003)* followed by anti-rabbit IgG. Immunoreactive bands were visualized with diaminobenzidine. TLC and immunostaining were done, as described previously *(Kusunoki et al., 1992)*.

Results

Samples

Anti-ganglioside antibody and TNF-α titers are summarized in Table 3.

Effects of GBS serum samples on extended neurites and on preserved neurons and proliferated Schwann cells in monolayer culture

GBS serum containing IgG anti-GalNAc-GD1a antibodies added to sufficiently elongated neurites damaged both myelin sheaths and Schwann cells, producing debris which was distributed along the axons (Fig. 1B–D) and small vacuoles (Fig. 1C). The most extensive damage was induced by serum from patients 1 and 3 (Fig. 1B), lesser damage being induced by serum from patient 2 (Fig. 1C), while serum from patient 4 induced only slight changes (Fig. 1D). Immunohistochemical analysis, however, showed that the axons had remained intact and that the debris originated from Schwann cells or myelin sheaths (middle and lower panel of Fig. 1B,C). Purified IgG anti-GalNAc-GD1a antibodies and TNF-α with inact-HS caused some damage to neurite myelin, producing debris along the axons (Fig. 1G,H). Immunohistochemical analysis showed intact axons and slightly damaged myelin (middle and lower panels of Fig. 1G,H).

The addition of noninactivated GBS serum to well-proliferated Schwann cells (Fig. 2, upper panel) and preserved neurons (Fig. 2, middle panel) in monolayer culture induced many small vacuoles in the Schwann cells (Fig. 2B,C), or caused rounding, indicative of slight cell detachment from the coated cover slips (data not shown, patients 4 and 5) and resulted in neuronal necrosis as evidenced by ballooning vacuoles (Fig. 2F,G). Purified IgG anti-GalNAc-GD1a antibodies elicited vacuoles in Schwann cell monolayers and in some preserved neurons (Fig. 2Gb). TNF-α had no effect on neurons, even after 14 days (Fig. 2Tb), but did cause Schwann-cell rounding after 7 days (Fig. 2Ta). No Schwann cells remained 10–14 days after the addition of TNF-α (Fig. 2Tb).

GBS serum with IgG anti-GM1 and anti-GD1b antibodies, and a high titer of TNF-α (patients 6 and 7), also damaged the myelin sheath in some regions of sufficiently elongated neurites, but immunohistochemical analysis did not show any myelin damage or vacuolation (Fig. 1E). Many small vacuoles were induced in well-proliferated Schwann cells (Fig. 2D), but there were no morphological changes in preserved neurons (Fig. 2H). Rabbit serum with a high titer of IgG anti-GalNAc-GD1a antibodies induced only minor or no damage in extended neurites, mature neurons, and Schwann cells. HS, serum from patient 1 following passage over Sepharose-bound GalNAc-GD1a (adsorbed serum), and serum from the same patient after recovery (Pt.1–6m) induced no damage to myelin sheaths, Schwann cells, or preserved neurons (Figs. 1A,F and 2A,E).

Regeneration model

Noninactivated GBS serum containing IgG anti-GalNAc-GD1a antibodies (patients 1–4), purified IgG anti-GalNAc-GD1a antibodies, and TNF-α inhibited neurite extension from DRG (Kruskal–Wallis test and, post-hoc, Scheffé's method; patient sera, $p < 0.0001$; purified IgG anti-GalNAc-GD1a antibodies and TNF-α, $p < 0.001$), in comparison with inact-HS and serum from patient 1 after recovery (Pt.1–6m) in the former (Fig. 3A) and serum from patient 1 after passage over Sepharose-bound GalNAc-GD1a in the latter (Fig. 3B).

As judged morphologically and immunohistochemically, neurite extension, myelin formation, and Schwann cell proliferation were inhibited by noninactivated GBS serum (patients 1–4), purified IgG anti-GalNAc-GD1a antibodies, and TNF-α (Fig. 4B, rB, E, rE, F, rF). Schwann cells exhibited small vacuoles in the presence of serum from patients 1–3 (Fig. 4B). Myelin formation was mostly inhibited by TNF-α (Fig. 4rF). Axons were split into segments by serum containing IgG anti-GalNAc-GD1a antibodies (patients 1–3) (Fig. 4fB), but not by purified IgG anti-GalNAc-GD1a antibodies or TNF-α (Fig. 4fE, fF), although they were very thin and their density was decreased (Fig. 4fE, fF).

Table 3. Harmful effects of serum samples on neurites from DRG, neurons, and Schwann cells.

		Serum effects on mature cells				Serum effects in the regeneration model			
		On sufficiently extended neurites from whole DRG embedded in collagen		Addition of serum 2 weeks after initiation of dissociated monolayer culture		On neurites growing from DRG embedded in collagen		Addition of serum 2 days after initiation of dissociated monolayer culture	
Samples	Titres of IgG anti-GalNAc-GD1a (anti-GalNAc) and TNF-α	Myelin damage	Axonal damage	On preserved neurons	On proliferated Schwann cells	Inhibition of neurite elongation	Inhibition of Schwann cell proliferation	Neuronal decrease	Inhibition of Schwann cell proliferation
Patient 1	Anti-GalNAc (4+) TNF-α (3+)	(3+) Vacuole (+)	(–)	Vacuole (+)	Vacuole (+)	(3+) Axon split (+)	(3+)	(3+) Vacuole (+) Floating (2+)	(2+) Vacuole (+) Floating (+)
Patient 2	Anti-GalNAc (3+) Anti-asialo-GM1 (+) TNF-α (+)	(2+) Vacuole (+)	(–)	Vacuole (+)	Round shape (+)	(3+) Axon split (+)	(2+)	(3+) Vacuole (+) Floating (2+)	(1+) Vacuole (–) Floating (+)
Patient 3	Anti-GalNAc (4+) TNF-α (3+)	(3+) Vacuole (+)	(–)	Vacuole (+)	Vacuole (+)	(3+) Axon split (+)	(3+)	(3+) Vacuole (+) Floating (+)	(2+) Vacuole (+) Floating (+)
Patient 4	Anti-GalNAc (2+) TNF-α (+)	(±)	(–)	Not done	Round shape (+)	(1+) Axon no change	(1+)	(1+) Vacuole (–) Floating (±)	(±) Vacuole (–) Round shape (+)
Patient 5	Anti-GalNAc (3+) Anti-asialo-GM1 (+) TNF-α (not done)	Not done	Not done	Not done	Not done	Not done	Not done	(1+) Floating (+)	(±) Round shape (+)
Patients 6 and 7	Anti-GM1 (3+) Anti-GD1b (+) TNF-α (3+)	(3+) Vacuole (–)	(–)	Vacuole (–)	Vacuole (+)	(3+) Axon no change	(3+)	(3+) Vacuole (–) Floating (–)	(±) Vacuole (–) Round shape (–)
Purified anti-GalNAc	Anti-GalNAc (4+) TNF-α (–)	(2+)	(–)	Vacuole (+)	Vacuole (–)	(2+) Axon no change	(2+)	(1+) Vacuole (2+) Floating (±)	(±) Vacuole (+)
TNF-α	Anti-GalNAc (–) TNF-α (2+)	(2+)	(–)	Vacuole (–)	Round shape (+)	(1+) Axon no change	(2+)	(1+) Vacuole (±) Floating (–)	(2+) Vacuole (+) Round shape (+)
Rabbit serum	Anti-GalNAc (4+) TNF-α (–)	(±)	(–)	Vacuole (±)	Vacuole (–)	(2+) Axon no change	(2+)	(2+) Vacuole (–) Floating (+)	(±) Round shape (+)
Human serum	Anti-GalNAc (–) TNF-α (–)	(–)	(–)	Vacuole (–)	Vacuole (–)	(–) Axon no change	(1+)	(–) Vacuole (–) Floating (–)	Vacuole (–) Round shape (–)
Recovered patient 1 (Pt.1–6m)	Anti-GalNAc (–) TNF-α (–)	(–)	(–)	Vacuole (–)	Vacuole (–)	(–) Axon no change	(–)	(1+) Vacuole (–) Floating (–)	(–) Vacuole (–) Round shape (–)
The serum that passed through the column (adsorbed serum)	Anti-GalNAc (–) TNF-α (–)	(–)	(–)	Vacuole (–)	Vacuole (–)	(–) Axon no change	(–)	(1+) Vacuole (–) Floating (–)	(–) Vacuole (–) Round shape (–)

DRG, dorsal root ganglia; IgG, immunoglobulin G; TNF-α, tumor necrosis factor-alpha. Anti-GalNAc: anti-GalNAc-GD1a; the serum that passed through the column: the serum adsorbed with GalNAc-GD1a. Anti-ganglioside and TNF-α titers were determined by ELISA. Anti-ganglioside titers: >640× (4+); >300× (3+); 100–200× (2+); 50–100× (+). TNF-α titers: 300–500 pg/mL (3+); 200–300 pg/mL (2+); 100–200 pg/mL (+). Degrees of inhibition, damage, and decrease: almost all and >80% (3+); about 50% and significantly (2+); <30% and not significantly (1+).

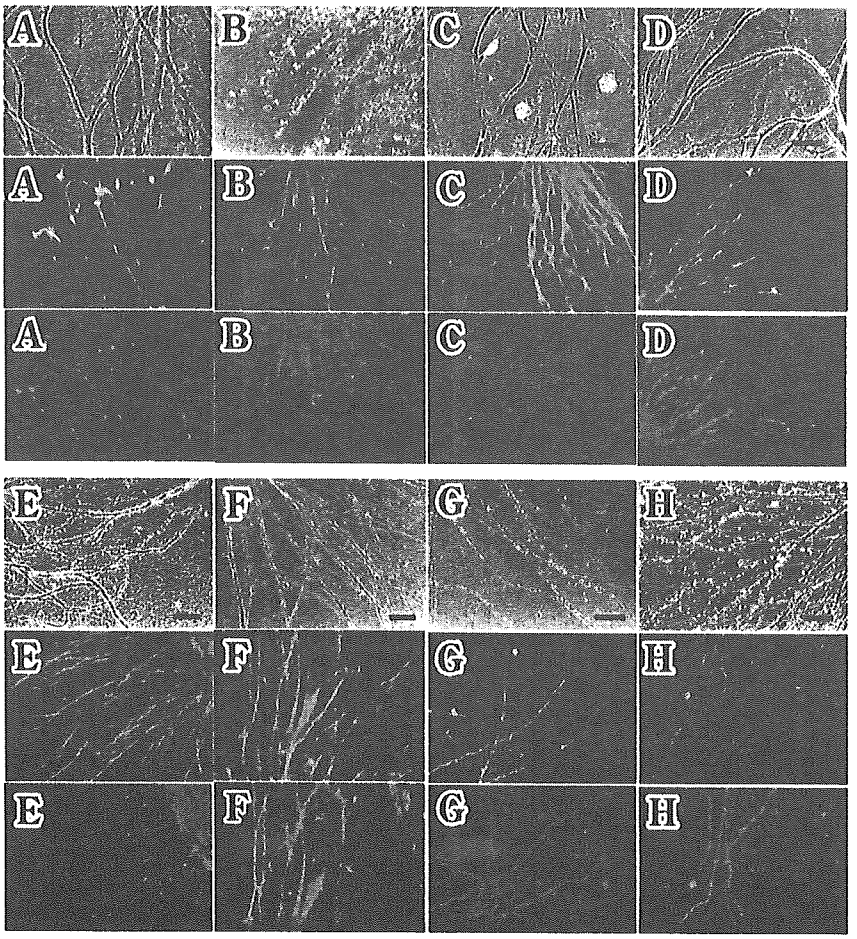

Figure 1. Effects of noninactivated patient serum (B–E) and purified immunoglobulin G (IgG) anti-GalNAc-GD1a antibodies or tumor necrosis factor-alpha (TNF-α) (G, H) on elongated and myelinated dorsal root ganglia (DRG) neurites. Serum samples were added to culture medium 10–14 days after the initiation of culture, and phase-contrast (upper panel) and fluorescence microscopy were performed after a further 7 days. Middle panel: rabbit anti-neurofilament antibody followed by fluorescence isothiocyanate anti-rabbit IgG. Lower panel: mouse anti-myelin basic protein (MBP) and mouse anti-S100 protein monoclonal antibody followed by Cy3 anti-mouse IgG. Serum from patients 1 (B), 2 (C), and 4 (D) induced myelin damage, but not axonal damage. Debris is distributed along axons and nonspecific myelin immunoreactivity is apparent in (B). Serum from patient 4 (D) caused less damage. Serum from patient 2 (C) induced morphological changes in Schwann cells but not in the myelin sheath. Serum from patient 6, containing IgG anti-GD1b and anti-GM1 antibodies, and a high TNF-α titer (E) damaged myelin in some regions, but immunohistochemical analysis showed that debris was not distributed along the axons. Purified IgG anti-GalNAc-GD1a antibodies (G) and TNF-α (H) in the presence of inact-HS caused damage to Schwann cells and the myelin of elongated neurites, the more severe damage to myelin being induced by TNF-α. (A) Noninactivated human control serum. (F) Serum from patient 1 after passage over Sepharose-bound GalNAc-GD1a. Bar = 50 μm.

Initially, in monolayer co-cultures, neurons thrived then deteriorated, whereas Schwann cells increased in the presence of NGF and FBS. In this system, non-inactivated GBS serum containing anti-GalNAc-GD1a antibodies caused statistically significant decreases in numbers of neurons after 3 and 6 days, as compared with inact-HS (Kruskal–Wallis test and, post-hoc, Scheffé's test, $p < 0.0001$) (Fig. 3C). Noninactivated serum from patients 1–3 facilitated cell detachment from laminin-coated cover slips. Purified IgG anti-GalNAc-GD1a antibodies and TNF-α induced greater decreases in neuronal numbers than control serum but not significantly (data not shown).

Schwann cells proliferated in the presence of control serum (inact-HS and serum from patient 1 after recovery), forming a whorl-like arrangement, whereas GBS serum containing IgG anti-GalNAc-GD1a antibodies (patients 1–5) inhibited Schwann-cell proliferation. Neurons and Schwann cells developed vacuoles, or Schwann cells became rounded, indicative of moderate detachment (Fig. 5B,C). Proliferation was moderate in the presence of serum from patients 4 and 5, but detachment was increased (Fig. 5D). On day 14, purified IgG anti-GalNAc-GD1a antibodies (Fig. 5G) inhibited Schwann cell proliferation and induced morphological changes in both neurons and Schwann

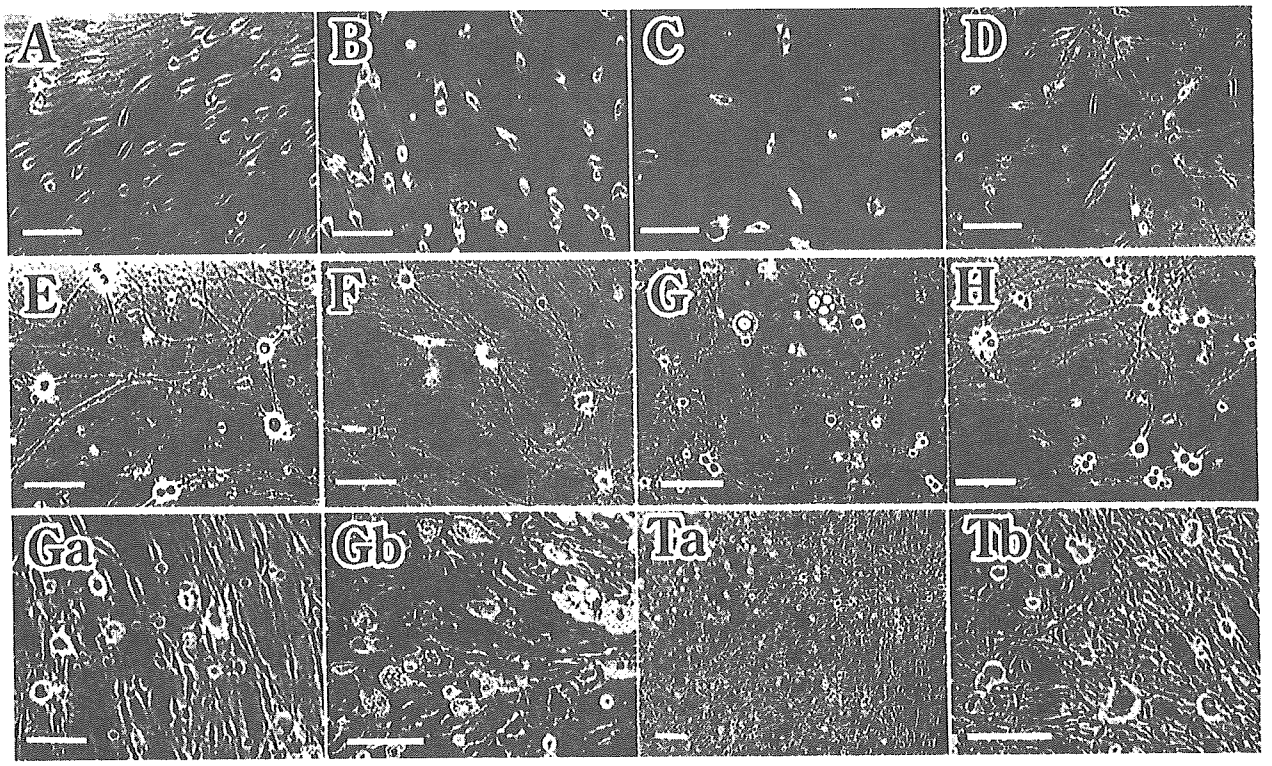

Figure 2. Effects of noninactivated patient serum on well-proliferated Schwann cells (upper panel) and preserved neurons (middle panel) when added to culture medium 10–14 days after the initiation of culture. Control: noninactivated serum from a healthy individual (A, E). Serum from patient 1 (B, F), patient 2 (G), and patient 3 (C) induced small vacuoles in Schwann cells and neurons. Serum from patient 6, containing IgG anti-GD1b and anti-GM1 antibodies and a high tumor necrosis factor-alpha (TNF-α) titer induced small vacuoles in Schwann cells (D) but not in neurons (H). Lower panel: effects of purified anti-GalNAc-GD1a antibodies and TNF-α. Purified IgG anti-GalNAc-GD1a antibodies had induced vacuoles in only a few neurons at day 7 (Ga), but by day 14 had induced small vacuoles in all of the preserved neurons (Gb). TNF-α induced Schwann-cell rounding at day 7 (Ta). There were no neuronal changes, even 14 days after the addition of TNF-α, while Schwann cells were absent (Tb). Bar = 50 μm.

cells. The cells had small vacuoles similar to those produced by serum from patients 1 and 3. Neurons were preserved in the presence of TNF-α, and large vacuoles were apparent in only a few of them, but Schwann cell proliferation was inhibited at day 14 (Fig. 5H), and cells that had proliferated were rounded at day 7 (data not shown).

Inactivated serum had lesser negative effects than noninactivated serum from individual patients, and serum from patients containing lower IgG anti-GalNAc-GD1a antibody titers had a lesser inhibitory effect on neurite outgrowth, myelin formation, and Schwann cell proliferation.

Noninactivated serum from patients 6 and 7 containing IgG anti-GM1 and anti-GD1b and TNF-α also inhibited neurite outgrowth (Fig. 3A), myelin formation, and Schwann cell proliferation, but did not cause immunoreactive neurofilaments to split or elicit vacuoles in Schwann cells (Fig. 4C, rC, fC). In monolayer co-cultures, these sera caused statistically significant decreases in numbers of neurons after 3 and 6 days as compared with inact-HS (Kruskal–Wallis and, post-hoc, Scheffé's tests, $p < 0.0001$) (Fig. 3C) but did not elicit small vacuoles in neurons (Fig. 5E); they also inhibited Schwann cell proliferation, but there was no detachment or vacuolation.

Rabbit serum containing IgG anti-GalNAc-GD1a antibodies inhibited neurite extension, myelin formation, and Schwann cell proliferation after 2 weeks of culture, in contrast to control rabbit serum (Kruskal–Wallis test, post-hoc, Scheffé's test, $p < 0.0001$) but axons remained intact. Rabbit serum containing IgG anti-GalNAc-GD1a antibodies inhibited Schwann cell proliferation in dissociated monolayer cultures, and facilitated detachment, but numbers of neurons were decreased to no greater extent than with control rabbit serum. No vacuoles were induced in neurons or Schwann cells during 6 days of culture. Serum from healthy human individuals (HS), serum from patient 1 after passage over Sepharose-bound GalNAc-GD1a with inact-HS, and serum from patient 1 after recovery did not induce any damage (Table 3).

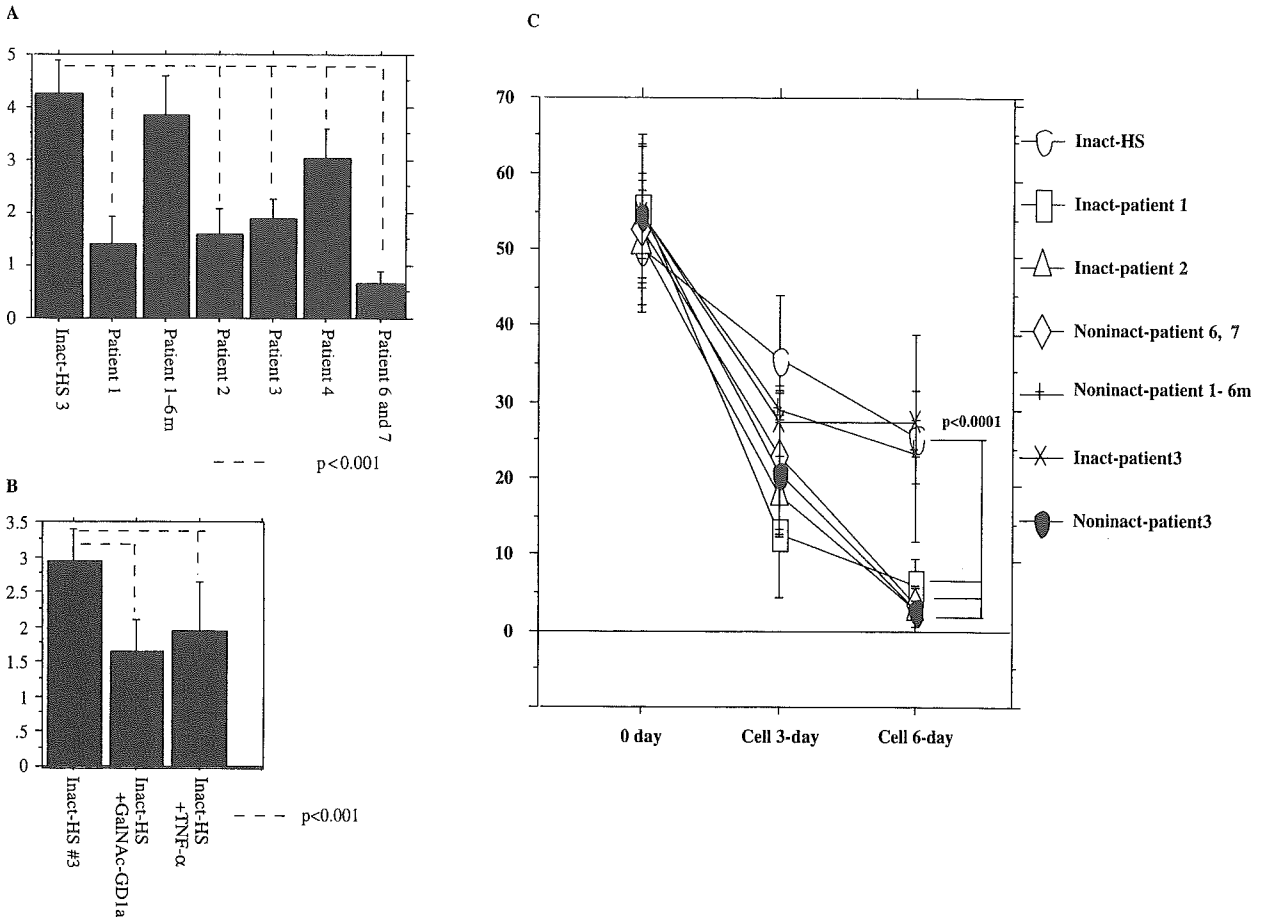

Figure 3. Regeneration model. Samples were added from the start of culture. Dorsal root ganglia (DRG) neurite outgrowth is shown as multiples of the short diameter of DRG cultured for 2 weeks in medium containing individual serum samples (A, B). (A) Noninactivated serum containing IgG anti-GalNAc-GD1a antibodies (patients 1–4) caused less neurite extension than expected in comparison with inactivated serum from a healthy individual (inact-HS) or noninactivated serum from patient 1 after recovery (Pt.1–6m) (Kruskal–Wallis, post-hoc, Scheffé's tests; $p < 0.0001$). Serum from patients with IgG anti-GM1 and anti-GD1b (patients 6 and 7) also inhibited neurite outgrowth. (B) Purified IgG anti-GalNAc-GD1a antibodies with inact-HS and TNF-α with inact-HS inhibited neurite outgrowth when compared with serum from patient 1 following passage over Sepharose-bound GalNAc-GD1a (adsorbed S.) with inact-HS (Kruskal–Wallis test, post-hoc, Scheffé's test, $p < 0.001$). (C) Decreases in numbers of monolayer-cultured neurons caused by additions of serum 2 days after the initiation of culture. Decreases were more marked in the presence of serum from Guillain-Barré syndrome patients with IgG anti-GalNAc-GD1a antibodies (patients 1, 2, and 3) or anti-GM1 and anti-GD1b antibodies (patients 6 and 7) than in the presence of serum from a healthy individual (HS). Serum from patients 1 and 2 was inactivated because noninactivated serum from these patients facilitated cell detachment from the laminin-coated cover slips. Noninactivated serum from patient 1 after recovery (Pt.1–6m), and inactivated serum from patient 3 (inact-patient 3) did not produce statistically significant decreases in numbers of neurons as compared with inactivated serum from a healthy individual (inact-HS) (Kruskal–Wallis test and, post-hoc, Scheffé's test, $p < 0.0001$).

GalNAc-GD1a in rat DRG and root

Co-cultures of DRG and nerve roots contained small amounts of GalNAc-GD1a, but DRG alone had none (Fig. 6).

Discussion

On the basis of the clinical characteristics of the axonal type GBS, we examined the effects of IgG anti-GalNAc-GD1a antibodies and TNF-α on DRG *in vitro*. The serum that includes IgM anti-GalNAc-GD1a was not used because that type of GBS showed different clinical characteristics from those with IgG anti-GalNAc-GD1a *(Kaida et al., 2000)*. The medium in which whole DRG and dissociated monolayers were cultured contained NGF because the DRG had been excised from 7-day-old rats which were easy to treat.

Our results show that serum containing IgG anti-GalNAc-GD1a antibodies is harmful to both axonal regeneration and neuronal preservation; to neuronal attachment to laminin-coated cover slips; and, possibly, to the cell membrane as evidenced by the presence of

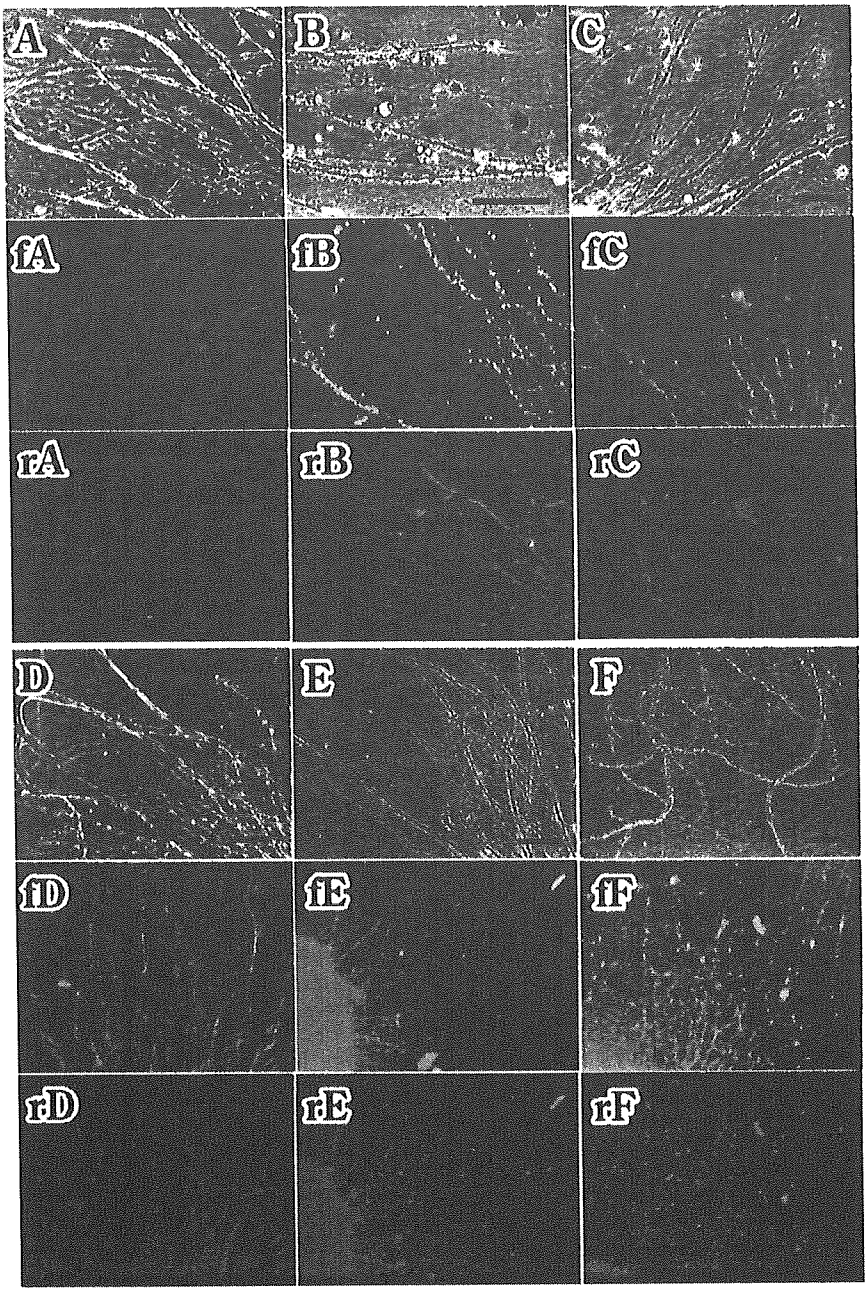

Figure 4. Effects of serum from patients on neurite elongation and myelin formation. Patient serum, purified anti-GalNAc-GD1a antibodies, or tumor necrosis factor-alpha (TNF-α) were added 2 days after the initiation of culture. After 14 days, cultures were observed by phase-contrast and fluorescence microscopy. Noninactivated serum from patient 3 (B) inhibited myelin formation in DRG. Vacuolated cells are apparent and debris is distributed along the axons (B) and myelin formation was inhibited (rB). Axonal splitting is shown by fluorescence isothiocyanate-labeled anti-neurofilament antibodies (fB). Noninactivated serum from patient 6 containing IgG anti-GM1 and anti-GD1b antibodies also inhibited Schwann cell proliferation and myelin formation (C, rC), and slight axonal splitting is visible (fC). IgG anti-GalNAc-GD1a antibodies purified from serum from patient 1 (E) together with inact-HS inhibited axonal elongation and damaged the myelin sheath (fE, rE). TNF-α with inact-HS (F) caused elongation of thin axons but inhibited myelin formation (fF, rF). In B, C, E, and F, nonspecific immunoreactive staining was increased. Control: inact-HS (A, fA, rA) and serum from patient 1 after passage over Sepharose-bound GalNAc-GD1a together with inact-HS (D, fD, rD). Bar = 50 μm.

small vacuoles. IgG anti-GalNAc-GD1a antibodies may be the cause of damage to neurons and axons in axonal type GBS during the early and recovery phases. We were unable to determine, however, whether IgG anti-GalNAc-GD1a antibodies induced tissue damage directly or indirectly via inhibition of NGF effects.

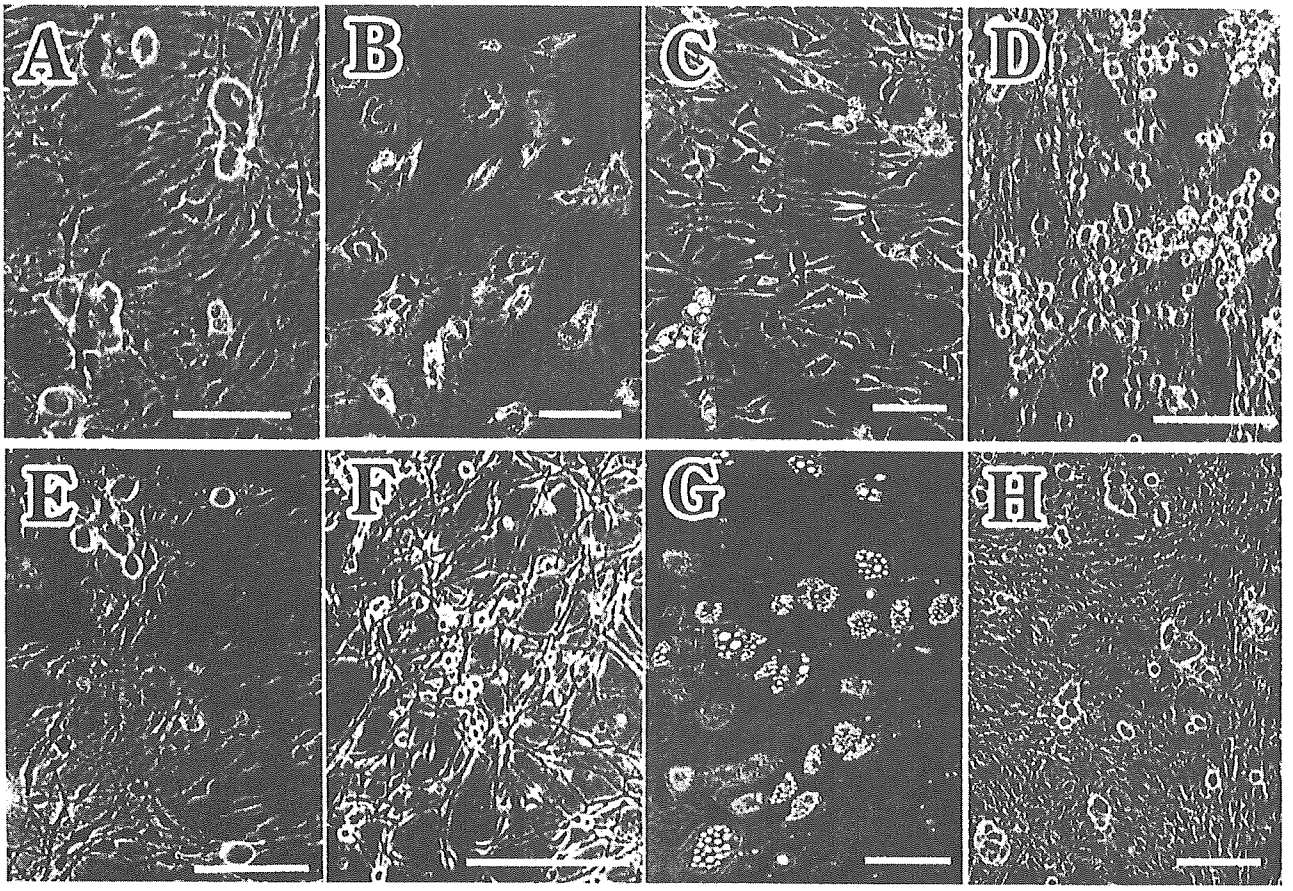

Figure 5. Effects of serum from patients, purified IgG anti-GalNAc-GD1a antibodies from patient 1, and tumor necrosis factor-alpha (TNF-α) on monolayers of dissociated cultured neurons and Schwann cells. Serum was added 2 days after the initiation of monolayer culture (regeneration model), and cultures were examined by phase-contrast microscopy after a further 7 days. Effects of inactivated serum from a healthy individual (inact-HS, Control, A), serum from patients 1 (B), 3 (C), and 5 (D), inact-HS with serum from patient 1 after passage over Sepharose-bound GalNAc-GD1a (Control, F), purified anti-GalNAc-GD1a antibodies from serum of patient 1 with inact-HS (G), and TNF-α with inact-HS (H). Control serum (A, F) allowed Schwann-cell proliferation and neuronal preservation. The morphology of neurons and Schwann cells changed in the presence of serum from patients 1 and 3 (B and C), and the cells had small vacuoles. Serum from patient 5 (D) caused rounding of Schwann cells, indicative of moderate detachment. Serum from patient 6 containing anti-GM1 and anti-GD1b antibodies (E) caused no morphological changes in neurons or Schwann cells. After culture for 14 days with purified IgG anti-GalNAc-GD1a with inact-HS (G), most neurons and Schwann cells had small vacuoles similar to those produced by serum from patients 1 and 3. TNF-α with inact-HS inhibited Schwann cell proliferation and large vacuoles were present in some remaining neurons (H). Bar = 50 μm.

Given that patient serum continued to show moderate TNF-α titers, and that TNF-α activity may have been decreased by inactivation, the contribution of TNF-α to cell damage was also significant. TNF-α may have had a major role in the negative effects produced in cultured myelin and Schwann cells because of the importance of cytokines in the pathogenesis of GBS *(Zhu et al., 1998)*. TNF-α induces demyelination and is toxic to oligodendrocytes in central nervous system diseases *(Selmaj et al., 1991)*, but its role in demyelination in the peripheral nervous system and in damaging Schwann cells remains controversial *(Mithen et al., 1990; Chandross et al., 1996)*. Our findings confirm the importance of TNF-α in the pathogenesis of GBS. The concept of there being an axonal type GBS derives from electrophysiological studies, and the present results independently suggest that in this form damage to Schwann cells or the myelin sheath might be induced in some regions of peripheral nerves.

Serum from GBS patients containing IgG anti-GM1 and anti-GD1b antibodies had the same effects on DRG as were caused by serum from patients containing IgG anti-GalNAc-GD1a antibodies, particularly as they related to myelin and Schwann cells. Serum with IgG anti-GM1 and anti-GD1b antibodies, however, did not cause neurofilament splitting, morphological

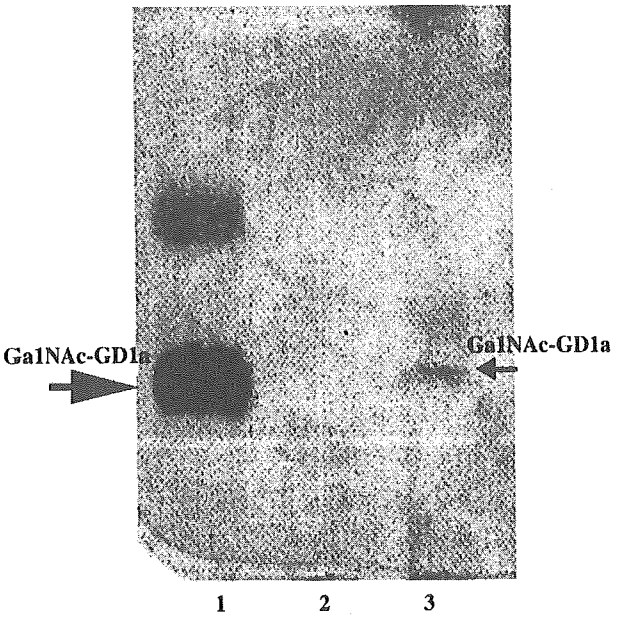

Figure 6. Thin-layer chromatography of the lipid fraction from rat dorsal root ganglion (DRG) and roots immunostained with rabbit IgG anti-GalNAc-GD1a antibodies followed by anti-rabbit IgG. The immunoreactive band was visualized by diaminobenzidine staining. Lane 1, GM1 ganglioside and GalNAc-GD1a markers. Lane 2, DRG. No bands are present. Lane 3, DRG and root mixture. The band has migrated to the position of GalNAc-GD1a (large arrow).

changes such as small vacuoles in neurons, or detachment. We suspect that IgG anti-GD1b and anti-GM1 antibodies, as well as IgG anti-GalNAc-GD1a antibodies, are harmful to Schwann cells, but assume that the effects seen on the myelin and Schwann cells were mainly caused by TNF-α. IgG anti-GD1a and anti-GM1 antibodies, however, are also involved in neuronal preservation and the inhibition of neurite elongation. As GM1 is located on the surface of neurons *(Tettamanti and Riboni, 1994)* and functions in the regulation of cell *(Spoerri et al., 1988)* and axonal *(Doherty et al., 1992)* growth, GalNAc-GD1a may function, in part, like GM1.

Rabbit IgG anti-GalNAc-GD1a antibodies had fewer effects than purified human IgG anti-GalNAc-GD1a antibodies, inhibiting only neurite elongation and Schwann cell proliferation in the embedded DRG culture system. The damage caused by patient sera containing IgG anti-GalNAc-GD1a antibodies to proliferated, mature Schwann cells or myelin therefore was most likely primarily due to the high TNF-α titer. This is because purified IgG anti-GalNAc-GD1a antibodies and rabbit serum contained little TNF-α, the former having been obtained after adsorption and the latter obtained after a long time after immunization. Although immunized rabbits had a very high IgG anti-GalNAc-GD1a antibody titer, no neuropathy developed, indicating that rabbit IgG anti-GalNAc-GD1a antibodies may not be pathogenic.

In conclusion, in the early stage of GBS, an increased TNF-α titer seems to play a major role in the destruction of myelin in the proximal and most distal regions of the nerve, with increased numbers of IgG anti-GalNAc-GD1a antibodies having a secondary role in neuronal or axonal membrane destruction. Such a pathogenesis would be likely to severely damage axonal function. In the later stages of the disorder, an increased IgG anti-GalNAc-GD1a antibody titer inhibits both the recovery of myelin sheath tissue and the axonal elongation. In addition, IgG anti-GalNAc-GD1a antibodies may negatively affect neuronal preservation. Most of our patients underwent repeated plasmapheresis 10–14 days after the onset of illness, and high titers of IgG anti-GalNAc-GD1a antibodies and TNF-α were adsorbed on the plasmapheresis columns. In those days, no patient received intravenous γ-globulin treatment. Early treatment is important for the recovery of GBS patients.

Acknowledgements

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MAJOR ARTICLE

Comprehensive Analysis of Bacterial Risk Factors for the Development of Guillain-Barré Syndrome after *Campylobacter jejuni* Enteritis

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Background. Guillain-Barré syndrome (GBS), a postinfectious autoimmune-mediated neuropathy, is a serious complication after *Campylobacter jejuni* enteritis.

Methods. To investigate the bacterial risk factors for developing GBS, genotypes, serotypes, and ganglioside mimics on lipo-oligosaccharide (LOS) were analyzed in *C. jejuni* strains from Japanese patients.

Results. Strains from patients with GBS had LOS biosynthesis locus class A more frequently (72/106; 68%) than did strains from patients with enteritis (17/103; 17%). Class A strains predominantly were serotype HS:19 and had the *cstII* (Thr51) genotype; the latter is responsible for biosynthesis of GM1-like and GD1a-like LOSs. Both anti-GM1 and anti-GD1a monoclonal antibodies regularly bound to class A LOSs, whereas no or either antibody bound to other LOS locus classes. Mass-spectrometric analysis showed that a class A strain carried GD1a-like LOS as well as GM1-like LOS. Logistic regression analysis showed that serotype HS:19 and the class A locus were predictive of the development of GBS.

Conclusions. The high frequency of the class A locus in GBS-associated strains, which was recently reported in Europe, provides the first GBS-related *C. jejuni* characteristic that is common to strains from Asia and Europe. The class A locus and serotype HS:19 seem to be linked to *cstII* polymorphism, resulting in promotion of both GM1-like and GD1a-like structure synthesis on LOS and, consequently, an increase in the risk of producing antiganglioside autoantibodies and developing GBS.

The gram-negative spiral bacterium *Campylobacter jejuni*, which is a major bacterial agent in diarrheal illnesses, has been recognized as the bacterium that most frequently triggers the postinfectious autoimmune-mediated neuropathy called Guillain-Barré syndrome (GBS) [1]. An epidemiological study showed that 1 of 3285 patients with *C. jejuni* enteritis developed GBS [2]. Why such a small number of patients with *C. jejuni* enteritis develop GBS is not clear. Penner serotyping showed that, in Japan and South Africa, GBS-associated strains were more commonly serotypes HS:19 and HS:41 than were enteritis-associated strains [3–5]. Furthermore, HS:2 and the HS:4-complex were the dominant serotypes of strains from patients with Fisher syndrome (FS) [5], a GBS variant presenting the triad of ophthalmoplegia, ataxia, and areflexia [6]. The clustering of strains into particular serotypes is a strong indication that the clonality of *C. jejuni* strains is specifically related to the development of GBS and FS. The actual serodeterminants, however, are still unknown [7]; therefore, use of Penner serotyping schema alone to clarify the critical factors for the development of neurological syndromes would be difficult. It is noteworthy that the clustering of GBS-associated and FS-associated strains into specific serotypes has not been

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seen worldwide and, in particular, has not been seen in Western countries [8, 9].

Most patients who develop GBS after *C. jejuni* enteritis have IgG autoantibodies in their blood that react with gangliosides (such as GM1, GD1a, and GQ1b) [10]. Many patients with FS have anti–GQ1b IgG autoantibodies that cross-react with GT1a [11, 12]. The *C. jejuni* lipo-oligosaccharide (LOS) is a major candidate for the producer of such autoantibodies [13–15], because its terminal sugar regions mimic the gangliosides GM1, GD1a, and GQ1b [16–18]. The frequency of the GM1 and GD1a epitopes on the LOS of GBS-associated strains is hypothesized to be a risk factor for the development of GBS [19, 20]. Acute motor axonal neuropathy and anti-GM1 antibodies developed in rabbits after inoculation with GM1-like LOS, which indicates that GM1 mimicry of *C. jejuni* LOS is a cause of GBS [14].

Most studies have failed to find a specific *C. jejuni* genotype that was associated with GBS and FS [9, 21–24]. Gilbert et al. [25] reported that *C. jejuni* has 7 classes (A–G) of LOS locus that are based on the organization of the 37 distinct genes found in the LOS biosynthesis loci of 20 strains. This LOS locus typing scheme should help in the identification of the gene content that is responsible for the development of GBS and FS. Godschalk et al. [15], who used clinical isolates from The Netherlands and Belgium, recently reported that the class A locus was overrepresented in GBS-associated strains, compared with enteritis-associated strains (9/17 [53%] vs. 3/21 [14%]), whereas all FS-associated strains had the class B locus (FS-associated strains, 4/4 [100%]; enteritis-associated strains, 7/21 [33%]). The high frequency of the class A locus in GBS-associated strains has been confirmed by Parker et al. [26], who used 16 GBS-associated strains from various countries, although the frequency of this locus in GBS-associated strains could not be compared with that in enteritis-associated strains from each country. Godschalk et al. [15] suspected that the frequent expression of a GM1-like LOS in class A strains and a GQ1b-like LOS in class B strains is responsible for the development of GBS and FS, respectively. Their findings, however, do not clarify which genetic difference leads to the presence of diverse ganglioside mimics (GM1 and GQ1b) in spite of the almost identical gene profiles in class A and B loci [27] or why class C is relatively rare (2/17 [12%]) in GBS-associated strains in spite of the expression of GM1-like LOSs in all 5 strains with the class C locus.

The sialyltransferase gene *cstII* has an Asn/Thr polymorphism at codon 51, which determines substrate specificity; *cstII* (Thr51) has only α-2,3-sialyltransferase activity and is termed "monofunctional" *cstII*, whereas *cstII* (Asn51) has both α-2,3- and α-2,8-sialyltransferase activities and is termed "bifunctional" *cstII* [27]. Both sialyltransferase activities are required for the biosynthesis of the GQ1b and GT1a epitopes on LOS, whereas only α-2,3-sialyltransferase activity is needed for the biosynthesis of the GM1 and GD1a epitopes (figure 1). These findings recently led to our discovery that *cstII* polymorphism is important for the development of GBS and FS after *C. jejuni* enteritis [29]. *cstII* (Thr51) is closely associated with GBS and anti-GM1 and anti-GD1a autoantibodies, and *cstII* (Asn51) is closely associated with FS and anti-GQ1b autoantibodies.

Some of the identified bacterial risk factors for the development of GBS are closely related to each other, especially ganglioside mimics and serotype [19], *cstII* gene content [19, 29], and LOS locus class [15]. Therefore, it is necessary to analyze the risk factors comprehensively in a larger number of clinical isolates. We first examined whether the clustering of GBS-associated and FS-associated strains into a specific LOS locus class would also occur with a large number of Japanese strains; we then analyzed LOS locus classes comprehensively in connection with Penner serotype, *cstII* polymorphism, and ganglioside-like LOSs, to identify risk factors for the development of GBS.

MATERIALS AND METHODS

Strains. From December 1990 to February 2004, 138 *C. jejuni* strains were isolated from patients with GBS ($n = 106$) or FS ($n = 32$), and these strains were used in the present study. Most of the strains were included in our previous study [5]. Two strains, OH4384 and OH4382, were obtained from patients with GBS who were siblings [17, 30], and the others were obtained from patients with GBS who were evenly distributed geographically [5]. Diagnosis of GBS or FS was based on published clinical criteria [31, 32]. A total of 103 strains were isolated from patients throughout Japan who had uncomplicated enteritis, and these strains served as controls. Penner serotypes were determined using the passive hemagglutination technique with a *Campylobacter* antisera "Seiken" Set (Denka Seiken) [5].

LOS locus classification and* cstII *polymorphism. We used a method similar to that of Godschalk et al. [15] to classify the LOS locus (A–F). The presence of each class-specific gene was investigated by polymerase chain reaction (PCR) (table 1). The primer pair used for *orf19d* amplification was the same as that used in the study by Godschalk et al. [15], whereas the other primer pairs were newly designed for the present study. Class G was not examined, because it is considered to be very rare. Strains were judged to be class A when *orf7a/b (cstII)* was present and *orf5IIb (cgtAIIb)* was absent. Similarly, strains were judged to be class F when *orf19d/f* was present and *orf17d* was absent. A single bacterial colony was suspended in 300 μL of sterile distilled water and boiled for 10 min. After centrifugation at 10,000 *g* for 1 min, the supernatant was used as the template in the PCR amplification. Amplification reactions were performed with a total volume of 20 μL, which contained 8 pmol of each primer, 0.4 μL of DNA lysate, 0.5 U of *Taq* DNA