disappear during repeated culture owing to the phase variation in homopolymeric G tract in LOS biosynthesis genes.[36] Another possibility is that FS patients were infected by several *C. jejuni* strains and the "causative" strain bearing ganglioside-like LOS had not been occasionally isolated. The presence of a GM1-mimicking epitope on the *C. jejuni* LOS[9,22,37] and the development of acute motor axonal neuropathy with anti-GM1 antibody after inoculation of rabbits with the GM1-like LOS[24] strongly suggest that anti-GM1 IgG antibody production is mediated by GM1 mimicry of *C. jejuni* LOS. To show that the principle of molecular mimicry provides a common pathogenesis of FS and GBS, it is necessary to establish the FS animal model by immunizing GQ1b-bearing LOSs of *C. jejuni* and *H. influenzae*.

Our serologic findings and the detection of GQ1b-like LOS in FS-related *H. influenzae* strains provide strong support for a relationship between FS and *H. influenzae*. This bacterium is classified as having capsulated (serotypes a to f) and uncapsulated (nontypable) strains. We determined the serotypes of the FS- and GBS-related *H. influenzae* strains and found all were nontypable. We cannot say that all the isolates were related to the development of FS and GBS because this bacterium is a major pathogen of respiratory infection, and patients with FS or GBS sometimes contract pneumonia after neuropathic onset, possibly owing to bulbar palsy. Our results, however, do indicate that uncapsulated strains are important in the development of *H. influenzae*-related FS and GBS, but whether *H. influenzae* is a major causative agent of GBS has still to be determined.[8,15,38,39] In our study, there was serologic evidence of this infection in only 3% of the GBS patients, a frequency similar to findings of previous studies.[8,15,39] Because all the studies, including the current one, used only serologic methods to test for antecedent *H. influenzae* infection, the seropositive frequency may have been underestimated owing to the low sensitivity of the assay.[8] A standardized, highly sensitive serologic method and a culture survey are needed to establish the frequency of *H. influenzae*-related GBS.

The LOSs of *C. jejuni* and *H. influenzae* vary considerably in the oligosaccharide structures on their outer cores, and previous studies showed that both bacteria commonly have sialylated LOSs.[40] Because ganglioside classification is based on the sialylation type, sialylation of a bacterial LOS may be the key to the development of FS and GBS after *C. jejuni* or *H. influenzae* infection. Three genes (*cst-I*, *-II*, and *-III*) in *C. jejuni*[41,42] and three genes (*lic3A*, *siaA*, *lsgB*) in *H. influenzae*[43,44] have been cloned for the sialylation enzyme. Whether the presence of the *cst-II* gene is a risk factor for developing GBS/FS after *C. jejuni* enteritis is not clear,[45,46] but it seems to be essential for the biosynthesis of a GQ1b-like LOS and therefore is closely related to the anti-GQ1b antibody in FS.[45] Variation in the LOS outer core could, however, be created not only by diverse gene contents.[36,47] We sequenced the genes that encode the glycosyltransferases involved in synthesis of the outer core of the LOS in *C. jejuni* CF93-6 (GenBank accession no. AY644679). The DNA sequence is 99% identical (6,041 of 6,047 bp) to the corresponding region in *C. jejuni* OH4384 (GenBank accession no. AF130984), which had been isolated from a patient with GBS who showed ophthalmoplegia[9,48] and expresses GT1a-like LOS similar to that of CF93-6.[9] The amino acid sequences of these glycosyltransferases involved in the addition of the *N*-acetylgalactosamine residue (CgtA), terminal galactose residue (CgtB), and sialic acid residues (Cst-II) are 100% identical for *C. jejuni* CF93-6 and OH4384. This suggests that gene alleles also are critical for the biosynthesis of variable ganglioside mimics. To determine the critical factor in the development and characterization of FS after *C. jejuni* or *H. influenzae* infection, the presence and polymorphism of sialyltransferase-encoding genes need to be investigated.

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Side Effects of Combined Therapy of Methylprednisolone and Intravenous Immunoglobulin in Guillain-Barré Syndrome

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Key Words
Guillain-Barré syndrome · Methylprednisolone · Intravenous immunoglobulin · Side effect

Abstract
Side effects were compared in 9 patients with Guillain-Barré syndrome treated with standard intravenous immunoglobulin (IVIg) only and in 9 treated with combined methylprednisolone and IVIg therapy. Headache occurred in 2 in both groups, indicative that pre-infusion with steroids does not prevent headache. Transient liver function disturbances were present in 2 patients of the former group and in 6 of the latter.

Additional effects of methylprednisolone in the standard treatment with intravenous immunoglobulin (IVIg) for Guillain-Barré syndrome (GBS) were reported in a randomized control study [1]. Preliminary results indicated that there was a favorable effect of combined therapy on functional improvement after 4 weeks and on the median time to independent walking. After September 2001, when the Peripheral Nerve Society meeting was held [2], we therefore switched the first-line therapy for patients with GBS from IVIg alone to the combined therapy. To determine whether pre-infusion with steroids reduces the complications caused by IVIg treatment [3], we compared the side effects experienced by patients treated with the combined therapy and those treated with standard IVIg therapy.

Patients and Methods

Eighteen consecutive patients admitted to the Dokkyo University School of Medicine Hospital who fulfilled the criteria for GBS [4] were studied. Nine, admitted before the results of combined therapy were communicated, had been treated with IVIg alone (standard IVIg group), the other 9 had been treated with methylprednisolone and IVIg (combined therapy group; table 1). All the patients received 400 mg/kg of sulfonated human immunoglobulin (Venilon®-I, Teijin, Tokyo, Japan) intravenously daily for 5 days. In the combined therapy group, 500 mg of methylprednisolone (Solu-medrol®, Pharmacia, Tokyo, Japan) was given intravenously over a 2-hour period before IVIg treatment.

During the treatment period and 4 weeks after the final infusion, minor side effects were evaluated in terms of the appearance of physical symptoms and signs. Major effects were anaphylaxis, congestive heart failure, renal failure and thrombotic events. Hematological, serum chemistry and CSF findings were obtained routinely every week or every other week. Analysis of liver function disturbance was primarily based on the outcomes for alanine aminotransferase (ALT), the most liver-specific enzyme, and for γ-glutamyl transferase (γ-GTP), the most liver-sensitive enzyme, as in a previous study [5]. Neutropenia was defined as a cell count decreased below the lower limit of normal (<4,000/μl). CSF pleocytosis was defined as a high cell count, 10 cells/μl or more.

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Table 1. Clinical profiles of and side effects in patients with GBS treated with standard IVIg or combined therapy

	Standard IVIg group	Combined therapy group
Number of patients	9	9
Sex (male/female)	7/2	5/4
Median age, years	58 (24–76)	37 (20–73)
Clinical profiles		
Antecedent infectious illness		
Diarrhea	3	5
Upper respiratory tract infection	3	2
Electrophysiological subtype		
Acute motor axonal neuropathy	4	9
Acute inflammatory demyelinating polyneuropathy	3	0
Unclassified	2	0
Median scale of Hughes' disability score[1] before treatment	4	3
Autonomic failure	2[2]	0
Side effects	5	7
Liver function disturbance	2	6
Hot flush	0	4
Headache	2	2
Fever	0	1
Leukopenia	2	0
Hypertension	1	0
Malaise, chest discomfort	0	0
Nausea, vomiting	0	0
Skin rash, itch, tingling	0	0

Figures in parentheses are ranges.

[1] 1 = Minor symptoms or signs, capable of running; 2 = able to walk >5 m without assistance; 3 = able to walk >5 m with assistance; 4 = bedbound; 5 = assisted ventilation required.

[2] One each had tachycardia and orthostatic hypotension.

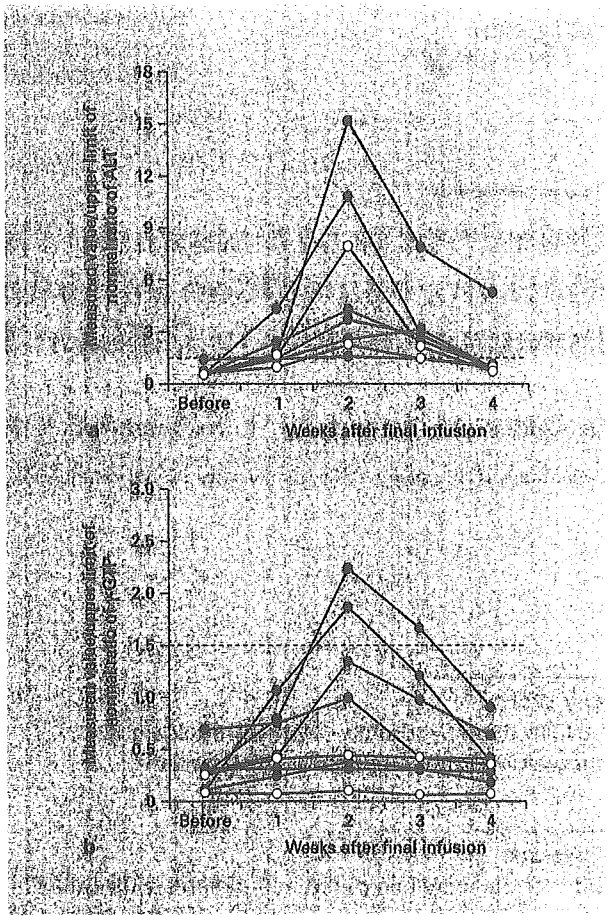

Fig. 1. Longitudinal changes of AST (**a**) and γ-GTP (**b**) before and after treatment in 8 patients with liver function disturbance. ○ = Standard IVIg; ● = combined methylprednisolone and IVIg therapy. Serum levels of ALT, γ-GTP, or both, are more than 1.5 the upper limit of normal, denoting liver function disturbance.

Results

Table 1 shows the side effects seen in each treatment group. No serious side effects were present in either group. Five of the 9 patients in the standard IVIg group experienced mild side effects compared to 7 of the 9 in the combined therapy group. Liver function disturbance, the most common side effect, was present in 2 of the former and 6 of the latter group (ALT was elevated in 8 patients in both groups and γ-GTP in 2 in the combined therapy group). The liver enzyme activities in both groups peaked during the second week after the final infusion, except for the ALT level of 1 patient in the combined therapy group, then decreased in the course of the illness (fig. 1).

Hot flush was seen in 4 patients in the combined therapy group during treatment, but it was mild and easily managed by limiting the infusion rate or by symptomatic treatment with an antihistamine. Headache, reported in 2 patients in both groups, developed after infusion and lasted for up to 3 days. Two patients treated with the combined therapy received an antalgic drug, but headache was short-lived in most of the patients. Neither meningeal irritation nor CSF pleocytosis was present in any of the 4 patients with headache. Leukopenia was present in 2 patients in the standard IVIg group. Their respective WBC counts dropped from 4,400 and 5,500/μl to 3,100

and 3,700/μl during the second week after the final infusion. Both patients were however asymptomatic and did not develop any infectious illness, and their counts returned to normal by the fourth week.

Discussion

IVIg and methylprednisolone were infused simultaneously in a published controlled study [2], but we tried giving methylprednisolone prior to IVIg to reduce side effects. The frequencies of mild side effects were higher in patients given the combined therapy than in those given the standard IVIg. In the controlled study of the combined therapy [1], the minor complications of hyperglycemia, urinary tract infection and hypertension were present in 21, 18 and 2%, respectively. Details of such mild side effects as liver function disturbance, hot flush, headache, fever and leukopenia have yet to be reported.

In a prospective study of 100 patients with GBS [5], the percentage of those with elevated liver function increased from 35% before to 69% shortly after receiving IVIg (Gammagard®). Another report on the side effects caused by IVIg given for neurological disorders found a significant elevation of liver enzymes after treatment [6]. Abnormal elevation depended on the IVIg preparation used (Alphaglobin®, Sandoglobulin® or Venimmun®), whereas there was no association with the underlying disease, age or gender. Although our study was conducted on a small number of patients, the frequency of liver function disturbance in the standard IVIg group tended to be low, possibly because of the difference in the IVIg preparations used. In contrast, the frequency of liver function disturbance in the combined therapy group was higher. Because liver function disturbance is rare in patients who receive high-dose steroid pulse therapy [7], the frequency of transient disturbances may have been increased by the addition of methylprednisolone. However, none of the patients experienced a clinical illness associated with active hepatitis, and after 2 months, liver function disturbance had disappeared spontaneously.

Hot flush was present in half the patients given the combined therapy. The frequency of headache was the same in both groups. The mechanism of IVIg-induced headache is not clear, but there seems to be a continuum between headache and the development of aseptic meningitis [8] which may be due to a hypersensitivity reaction to the entry of immunoglobulin into the subarachnoid space [9]. Pre-infusion with steroids may not be effective for preventing these symptoms.

Leukopenia develops in some patients treated with IVIg [6, 10], but we did not find it in the patients in our combined therapy group. Because steroids have the potential to induce leukocytosis, the addition of methylprednisolone might prevent reduced WBC counts. Similar side effect analyses as required by the recent Dutch trial are needed to confirm our observations.

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PAPER

Intractable chronic inflammatory demyelinating polyneuropathy treated successfully with ciclosporin

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Background: Chronic inflammatory demyelinating polyneuropathy (CIDP) is a heterogeneous disorder and both clinical course and response to treatment vary widely. Because of the propensity for relapse, CIDP requires maintenance therapy after the initial response to treatment. There is no consensus regarding this in the published literature.

Present report: A patient with CIDP was treated with oral prednisolone and cyclophosphamide pulse therapy but required repeated plasma exchange and intravenous immunoglobulin (IVIg). Treatment with ciclosporin freed the patient from repeated IVIg administration. Therapeutic responses in 14 subsequent cases including three patients who showed improvement with ciclosporin are also presented along with an algorithm of the authors' suggested protocol for treatment.

Conclusion: Ciclosporin should be considered for patients with intractable CIDP who require repeated IVIg.

Chronic inflammatory demyelinating polyneuropathy (CIDP) is characterised by hyporeflexia or areflexia and progressive or relapsing motor and/or sensory dysfunction of more than one limb, developing over at least two months.[1] Randomised controlled trials have shown beneficial short term responses to steroids,[2] plasma exchange (PE),[3 4] and intravenous immunoglobulin (IVIg),[5–8] but there is little information available on the effects of long term therapy. Therapy for CIDP should be individualised based on cost, availability, and potential adverse effects. IVIg and PE are expensive therapies, and protracted use of steroids is accompanied by a variety of side effects.

Ciclosporin is an immunosuppressive agent widely used in organ transplantation and certain autoimmune diseases. Reports suggest that it is effective for patients with CIDP who have experienced relapse or have failed to respond to other treatments.[9–11] Here we report a patient with pure motor CIDP who showed no improvement with prednisolone and cyclophosphamide pulse therapy but was successfully and safely treated with ciclosporin. Therapeutic responses of and outcomes for 14 subsequent patients are also presented. The aim of our study was to develop a provisional treatment strategy for CIDP based on published clinical trials and our own clinical experience.

CASE REPORT

A 34 year old man developed weakness in his left arm in February 1998. One month later, he had weakness in his right arm. The symptoms gradually worsened and in May 1998 he could no longer walk without support because he was easily fatigued. Cranial nerve examination revealed no abnormalities. On the Medical Research Council scale, muscle strength was grade 3 for the proximal and grade 2 for the distal muscles of the upper limbs. In the lower limbs, muscle strength was grade 4 for both the proximal and distal muscles. There were no tendon jerks or glove and stocking type paresthesias. His sense of touch, pain, vibration, and position was normal. The neuropathy impairment score (NIS) (previously called the neurological disability score[2]) for the right side was 48 and for the left side 51. (Based on the report by Dyck *et al*[2] the NIS was evaluated by sum score of the impairment of cranial nerves, muscle weakness, reflexes, and sensation: 0 = deficit, 1 = mild deficit, 2 = moderate deficit, 3 = severe deficit, and 4 = complete absence of function or severe deficit. Total score for maximum severity = 120.)

The results of the nerve conduction study are shown in table 1. In accordance with the diagnostic criteria for CIDP,[1] both the median and ulnar nerves had decreased motor conduction velocity. The left peroneal nerve had a partial conduction block. Abnormal temporal dispersions were noted in the segments between the wrist and elbow for both ulnar nerves and between the ankle and the segment below the fibular head for the right peroneal nerve. Both the median and posterior tibial nerves had prolonged distal latencies, and in the median and posterior tibial nerves the minimal F wave latencies were prolonged. Sensory nerve conduction studies showed no abnormalities. These findings met the demyelination criteria for CIDP.[1]

On cerebrospinal fluid examination the protein level was raised (99 mg/dl) with 1 cell/μl. The patient was diagnosed as having "probable CIDP" because of the clinical and physiological findings, based on the criteria published by the American Academy of Neurology AIDS Task Force.[1]

Figure 1 shows each treatment and response. He underwent six sessions of PE (every other day) starting on 23 June 1998. His strength improved rapidly after the first PE, and after the sixth he could walk without support. His NIS decreased to 6 (right) and 5 (left). Steroid therapy consisting of a daily dose of 60 mg (1 mg/kg) of oral prednisolone was started on 1 July 1998. After 1 month the dose was reduced by 5 mg every week. While the drug was being tapered off, on 20 October 1998, at a dose of 20 mg, limb weakness recurred.

Abbreviations: CIDP, chronic inflammatory demyelinating polyneuropathy; IVIg, intravenous immunoglobulin; NIS, neuropathy impairment score; PE, plasma exchange

Table 1 Nerve conduction study results

	Median nerve			Ulnar nerve			Peroneal nerve			Posterior tibial nerve			Sural nerve		
	Normal limit	Right	Left	Normal limit	Right	Left	Normal limit	Right	Left	Normal limit	Right	Left	Normal limit	Right	Left
Motor nerve conduction study															
Motor nerve conduction velocity (m/s)	>48.0	24.0	27.0	>48.0	38.0	36.0	>39.0	35.0	34.0	>38.0	38.0	38.0			
Distal latency (ms)	<4.5	8.7	6.3	<3.5	3.4	3.3	<6.2	7.5	7.7	<5.6	7.1	7.2			
Distal CMAP amplitude (mV)	>4.4	5.6	4.6	>4.6	5.8	4.3	>1.2	4.8	3.7	>5.0	5.1	4.6			
P/D ratio of CMAP amplitude		0.88	0.89		0.79	0.76		0.78	0.76		0.87	0.85			
P/D ratio of CMAP duration		1.11	1.07		1.16	1.20		1.21	1.11		1.12	1.17			
Minimum F-wave latency (ms)	<29.5	46.7	51.0	<29.1	ND	ND	<52.9	ND	ND	<52.7	73.5	76.0			
Sensory nerve conduction study															
Sensory nerve conduction velocity (m/s)	>44.0	48.0	50.0	>42.0	42.0	44.0							>40.0	48.0	47.0
SNAP amplitude (μV)	>11.1	28.4	29.8	>7.8	17.1	25.1							>3.0	16.0	10.2

The amplitude and duration of the initial negative phase were measured for the CMAPs.
CMAP, compound muscle action potential; ND, not done; P/D, proximal versus distal; SNAP, sensory nerve action potential.

He underwent five sessions of PE again (every other day), and the dose of prednisolone was increased to 60 mg. Although the combination of PE and prednisolone reduced his limb weakness, he had another relapse four weeks after the last PE.

Since the patient did not respond to steroids, we tried cyclophosphamide pulse therapy. From 12 December onwards he received 1.8 g (1 g/m^2) cyclophosphamide intravenously in accordance with the therapeutic regimen reported by Good *et al*.[12] He remained relapse free for four months, but in April 1999 he experienced relapses again. We adjusted the cyclophosphamide dose, in increments of 25% according to the protocol, to achieve transient reduction of the white blood cell count to 1500–2000 /μl. Although the dose was increased to 2.2 g (1.25 g/m^2) and then to 2.8 g (1.6 g/m^2), he continued to have relapses in May and June. PE was frequently required to alleviate the symptoms.

In May 1999, we stopped cyclophosphamide and replaced it with ciclosporin. Initially, 300 mg (5 mg/kg per day) of ciclosporin (Sandimmun, Sandoz, Basel, Switzerland) was given in two divided doses. From 12 June 1999 it was reduced to 250 mg, which corresponds to a serum concentration of between 150 and 200 ng/ml. Because the maximal effect of ciclosporin occurs after several weeks of treatment, the patient was given 25 g (0.4 g/kg) human immunoglobulin (Kenketsu glovenin-I-Nichiyaku, Nihon Pharmaceutical, Tokyo, Japan) intravenously daily for five days from 18 June onwards. His strength greatly improved four days after the last infusion, and eight days later, he could walk unaided. On 1 July he again developed weakness. As he could not stand, another course of IVIg was given for five days, starting on 12 July. Again his strength improved, and in August he could walk more than 100 m without needing to rest. Although improved strength was maintained, he was given 25 g IVIg (0.4 g/kg) daily for five days from 9 August onwards and 65 g (1 g/kg) human immunoglobulin each on 8 September and 6 October 1999.

In May 2000, his medication was switched from Sandimmun to Neoral (200 mg; Sandoz, Basel, Switzerland), a new formulation of ciclosporin based on a microemulsion drug delivery system. He had no further relapses for 27 months after the last treatment with IVIg. The dose of ciclosporin was slowly tapered and, on the patient's request, it was stopped on 30 January 2002. On 22 February hand weakness reappeared; 200 mg daily of ciclosporin was started again and IVIg was given daily for five days from 6 March onwards. His grip strength improved rapidly. The strength of all four limbs gradually increased and by August 2002 all weakness disappeared. Since restarting ciclosporin there have been no further relapses, the patient's condition having been stable for 20 months. At the last follow up evaluation in April 2004, he had normal strength in all limbs. The motor nerve conduction velocities in both median, ulnar, and right tibial nerves had increased, and the compound muscle action

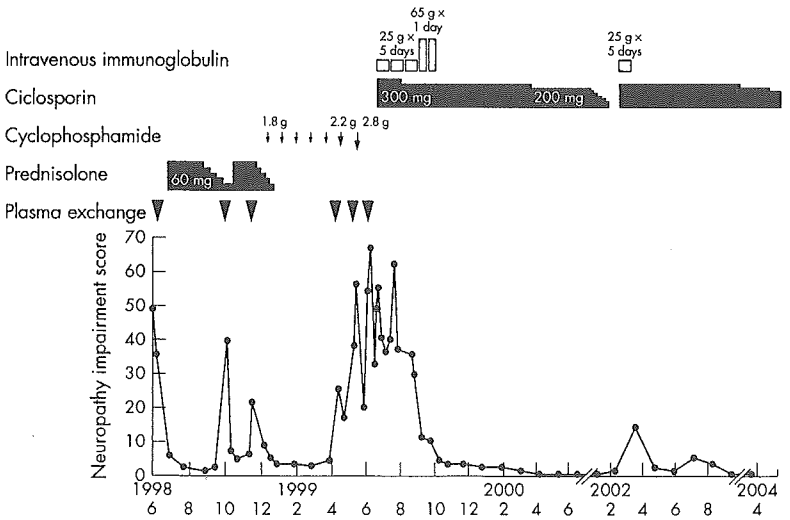

Figure 1 Clinical course: the patient did not respond to prednisolone and cyclophosphamide pulse therapies, but the neuropathy impairment score decreased (see right side of graph) during treatment with ciclosporin in combination with intravenous immunoglobulin.

potential amplitude had increased in both the tibial nerves and the left ulnar nerve. He is still taking ciclosporin, 160 mg/day, and the serum concentration is between 100 and 150 ng/ml. Except for hypertrichosis, there have been no side effects.

THERAPEUTIC RESPONSES OF AND OUTCOMES FOR 15 CONSECUTIVE PATIENTS WITH CIDP

Table 2 shows the therapeutic responses of and outcomes for 15 consecutive patients with CIDP, including the patient described above, who were treated with our proposed protocol as described below (fig 2). All patients were admitted to Dokkyo University School of Medicine Hospital between 1998 and 2003. All responded to IVIg or PE as the initial therapy, but four (patients 9–12) did not improve after being given steroids and a second line maintenance therapy was required.

Patient 9 did not respond to steroids and IVIg was frequently required. Subsequently, cyclophosphamide pulse therapy was given monthly for six months, but it was also unsuccessful. Ciclosporin was substituted for cyclophosphamide with no clear beneficial response. We considered using tacrolimus, and therapy was started with a daily dose of 3 mg. It had no beneficial effect and IVIg was given again.

Patient 10 had predominant motor dysfunction and responded rapidly to IVIg in combination with steroids, but relapse occurred while prednisolone was being tapered off. The steroid therefore was switched to ciclosporin. A second course of IVIg was given four weeks after the first, and the patient has been stable for nine months.

Patient 11 was given oral prednisolone after IVIg treatment. There was an initial response to prednisolone, but relapse occurred while it was being tapered off and diabetes mellitus developed. Although repeated IVIg produced transient improvement, relapse occurred again one month after the last IVIg. Treatment with ciclosporin was started, after which IVIg was no longer necessary. Since the introduction of ciclosporin there have been no further relapses, the patient's condition having been stable for 20 months.

Patient 12 had motor and sensory dysfunction, responded temporarily to IVIg, but limb weakness persisted. Despite treatment with prednisolone and PE, weakness remained. IVIg was given monthly for three months and ciclosporin started. The patient's strength gradually improved, and there have been no relapses for 18 months.

In all four patients there were no serious side effects of ciclosporin.

DISCUSSION

Our patient with CIDP did not respond to steroid and cyclophosphamide pulse therapies and was successfully treated with ciclosporin. Relapse after ciclosporin had been tapered off indicated that ciclosporin was effective and necessary as a maintenance therapy. The patient was young with selective motor involvement, no sensory symptoms or signs, and normal findings on sensory conduction studies. Four patients with a pure motor form of CIDP were reported by Sabatelli *et al*.[13] Selective involvement of motor fibres was suggested by the absence of abnormal sensory symptoms or signs and normal findings in an electrophysiological study of sensory fibres and in a sural nerve biopsy. Sabatelli's patients had a younger age of onset (3–29 years), and the clinical course was relapsing-remitting. All four patients did not

Table 2 Responses of and outcomes for 15 CIDP patients after treatment with the proposed standardised protocol

Patient	Age of onset/sex	Initial therapy and response	Maintenance therapies and responses				Outcomes*	NIS (severity)†	
			1	2	3	4		Before treatment	After stabilisation and remission
1‡	34/M	Plasma exchange	Oral prednisolone	Cyclophosphamide pulse	Ciclosporin		Stabilised for 26 months	51	0
		Temporarily responded	Temporarily responded, relapsed during tapering	Unchanged	Responded				
2	47/M	Plasma exchange	Oral prednisolone	No therapy required			Complete remission (40 months)	42	0
		Temporarily responded	Responded						
3	59/F	Plasma exchange	Oral prednisolone	No therapy required			Complete remission (34 months)	39	0
		Temporarily responded	Responded						
4	62/M	Plasma exchange	Oral prednisolone	No therapy required			Complete remission (32 months)	47	0
		Temporarily responded	Responded						
5	46/M	IVIg	Oral prednisolone	No therapy required			Complete remission (29 months)	36	0
		Rapidly responded	Responded						
6	52/M	IVIg	Oral prednisolone	No therapy required			Complete remission (28 months)	28	0
		Rapidly responded	Responded						
7	44/F	IVIg	Oral prednisolone	No therapy required			Complete remission (24 months)	46	0
		Slowly responded	Responded						
8	11/M	IVIg	Oral prednisolone	No therapy required			Complete remission (18 months)	30	0
		Rapidly responded	Responded						
9	53/M	IVIg	Oral prednisolone	Cyclophosphamide pulse	Ciclosporin	Tacrolimus	Stabilised for 12 months; maintained IVIg	66	12
		Temporarily responded	Unchanged, relapsed during tapering	Unchanged	Unchanged	Unchanged			
10	45/F	IVIg	Oral prednisolone	Ciclosporin			Stabilised for 9 months; maintained IVIg; no further relapses	37	6
		Temporarily responded	Temporarily responded, relapsed during tapering	Responded					
11	34/F	IVIg	Oral prednisolone	Ciclosporin			Stabilised for 20 months; no IVIg required; no further relapses	45	7
		Temporarily responded	Temporarily responded, relapsed during tapering	Responded					
12	65/F	IVIg	Oral prednisolone	Ciclosporin			Stabilised for 18 months; no IVIg required; no further relapses	48	4
		Temporarily responded	Unchanged, progressed	Responded					
13	19/F	IVIg	Oral prednisolone				Stabilised for 12 months; no IVIg required	31	4
		Rapidly responded	Responded						
14	45/M	IVIg	Oral prednisolone				Stabilised for 11 months; no IVIg required	42	6
		Rapidly responded	Responded						
15	37/M	IVIg	Oral prednisolone				Stabilised for 6 months; no IVIg required	37	3
		Rapidly responded	Responded						

*Duration of remission since steroids were stopped.
†Neurological impairment score; previously called the neurological disability score (described by Dyck PJ *et al*[7]); sum score of maximum severity = 120.
‡Patient in case report.
IVIg, intravenous immunoglobulin.

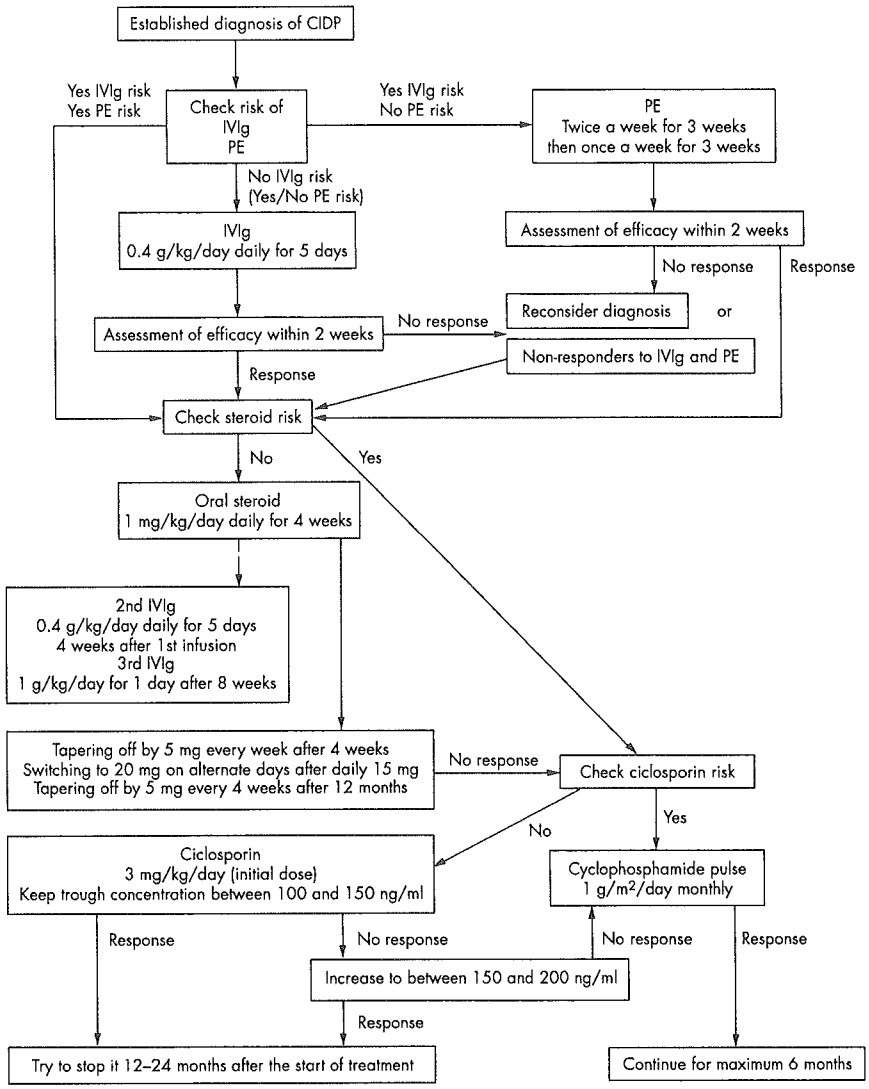

Figure 2 Proposed protocol for chronic inflammatory demyelinating polyneuropathy (CIDP) treatment. IVIg, intravenous immunoglobulin; PE, plasma exchange.

respond to steroids but showed considerable improvement with IVIg. Our patient's clinical features were similar to theirs. Ciclosporin *should be considered* for treatment resistant CIDP.

Successful treatment of CIDP with ciclosporin has been reported previously.[9–11] Mahattanakul *et al*[11] started ciclosporin at 3–5 mg/kg per day in eight patients, three of whom either improved or stopped taking prednisone. The authors reported that ciclosporin might prove an ideal long term treatment for young patients with relapsing CIDP, sparing them the side effects of steroids. Barnett *et al*[9] investigated the role of ciclosporin in the treatment of 19 patients with resistant CIDP who had not responded adequately to steroids, PE, IVIg, and other immunosuppressive therapies. The patients were divided into progressive and relapsing types based on the course of the disease and their response to therapy at follow up as graded by clinical and electrophysiological criteria. That study strongly indicated that ciclosporin was an effective therapeutic agent for patients with both types of resistant CIDP. They could not, however, identify any clinical characteristics which would predict a response to ciclosporin therapy.

CIDP is considered to be an immune mediated disorder based on its positive responses to immunotherapy, although the pathomechanisms are unclear. Antibodies have long been suspected of mediating nerve damage in CIDP because most CIDP patients respond to PE. Antibodies to the P0 myelin glycoprotein have been reported, which are capable of inducing experimental demyelination.[14] In contrast, endoneurial inflammatory changes due to T cell infiltration and macrophage associated demyelination during the active phase are often seen in nerve biopsy specimens.[15 16] Ciclosporin acts by inhibiting T cell dependent immune responses[17 18] and produces its immunosuppressant effects by inhibiting calcineurin mediated dephosphorylation of the nuclear factor of activated T cells bound to cyclophilin. Although pathomechanisms in CIDP are not clear, it may suppress responses of helper T cells by inhibiting the production of interleukin-2 and interferon-γ and preventing further recruitment and activation of macrophages.

PROVISIONAL TREATMENT PROTOCOL

On the basis of evidence from randomised controlled trials[2-8 19 20] and our own clinical experience, we have established a treatment protocol for CIDP (see fig 2). A randomised crossover study comparing IVIg with PE showed they were equally effective for treating CIDP.[19] A recent randomised crossover trial compared IVIg with oral prednisolone.[20] The outcome measure showed slight but not significantly more improvement with IVIg than with prednisolone. The duration of treatment, however, was relatively short for a chronic disorder that generally requires treatment for many months or years. Steroid therapy is inexpensive and readily available, but its benefits take as long as two months to appear.[20 21] In contrast, IVIg and PE provide rapid stabilisation and improvement, often within the first week of therapy.

In the treatment of CIDP, a distinction needs to be made between initial and maintenance therapy. Except in the presence of IgA deficiency, renal failure, and vascular disease or cardiac insufficiency complications, IVIg is generally well tolerated and easy to administer; therefore, IVIg rather than PE should be the initial therapy. For patients at risk with IVIg, PE is the preferred initial therapy. For those patients with illnesses of undetermined diagnosis, a trial IVIg or PE can resolve the nature of the immune-mediated disorder. A treatment algorithm for CIDP based on systematic review has been proposed.[22] It is recommend that patients with mild to moderate impairment and disability who do not have a pure motor form of CIDP or contraindication to steroid therapy should be started on prednisolone. Patients with moderate to severe impairment and disability in whom a quick response is needed or those with pure motor forms should be treated with IVIg. Most patients with CIDP improve after treatment with IVIg, but many experience relapses and require periodic administration of IVIg at intervals of several weeks to maintain improvement. The length of IVIg treatment needed is not yet clear. To decrease the frequency of IVIg administration, a trial of oral immunosuppressants should be offered to patients who have relapses.[5]

For maintenance therapy, steroids should be started in conjunction with IVIg or PE. Because the maximal effect of steroids may be delayed for two months or more after start of therapy,[20 22] additional IVIg courses should be provided. Second (0.4 g/kg per day daily for five days) and third (1 g/kg per day for one day) IVIg courses, respectively, have been given four and eight weeks after the first infusion. Because treatment for less than six months and rapid tapering of steroids may increase risk of relapse,[23] the steroid dose is slowly reduced over a 12 month period. There have been anecdotal reports of beneficial treatment with cyclophosphamide, ciclosporin, and mycophenolate but no randomised trials.[24] Based on our clinical experience, if patients have relapses after steroid reduction and require repeated IVIg or PE, ciclosporin should be considered. Published data on ciclosporin are limited to several small case series, and the response rates range from 40% to 90%.[9 11] If patients respond to treatment with ciclosporin, improvement generally starts after two months.[25] However, it has potential side effects such as nephrotoxicity, hypertension, nausea, oedema, hirsutism, headaches, and cramps, so patients with pre-existing severe renal impairment and hypertension should not be given ciclosporin. Nephrotoxicity, the most frequent side effect of ciclosporin, is usually dose dependent. It occurred in three of eight patients who began ciclosporin therapy on the high dose of 7 mg/kg per day or more and this necessitated reduction or cessation of the drug.[9] Ciclosporin therapy can be made safe and effective by starting with a dose of 3 mg/kg and by close clinical monitoring of the patient's serum ciclosporin and creatinine concentrations. Based on our experiences, serum trough concentrations should be kept between 100 and 150 ng/ml during the first six months of treatment. Patients who fail to respond to IVIg, PE, steroids, and ciclosporin may require other potentially effective immunotherapy such as cyclophosphamide,[12] tacrolimus,[26] interferon alfa[27] and beta,[28] mycophenolate,[29] or autologous stem cell transplantation.[30]

CIDP is a heterogeneous disorder and clinical course and response to treatment varies.[31] Also, there are several variants of the disease.[32-36] Whether each variant requires a specific treatment is not clear. Because it is impossible to predict in advance who will respond to which treatment, we use the standard therapeutic protocol to treat all patients with CIDP. The favorable findings in our patients, as well as those in previous reports,[9 11] support the need for a randomised controlled trial of ciclosporin for the treatment of CIDP. The beneficial effects of maintenance IVIg pulse therapy, reported by Hahn *et al*,[5] also require confirmation by long term trials. Larger studies may determine which subgroups of patients will respond to a particular therapeutic regimen.

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Patient consent was obtained.

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Ganglioside mimicry as a cause of Guillain–Barré syndrome

Nobuhiro Yuki and Masaaki Odaka

Purpose of review

Campylobacter jejuni is the most frequent agent of antecedent infection in an axonal variant of Guillain–Barré syndrome, acute motor axonal neuropathy, and anti-GM1 or anti-GD1a IgG antibody is also associated with acute motor axonal neuropathy. Molecular mimicry has been found between human GM1 ganglioside and the lipo-oligosaccharide of *C. jejuni* isolated from an acute motor axonal neuropathy patient. Progress has been made in Guillain–Barré syndrome research, especially on acute motor axonal neuropathy subsequent to *C. jejuni* enteritis.

Recent findings

Sensitization of rabbits with *C. jejuni* lipo-oligosaccharide, as well as GM1, induced the production of anti-GM1 IgG antibody, and the subsequent development of acute flaccid paralysis. Pathological changes in rabbit peripheral nerves were identical to those seen in human acute motor axonal neuropathy. These findings provide conclusive evidence that molecular mimicry is a cause of human autoimmune disease. Ganglioside-like lipo-oligosaccharide is synthesized by sialyltransferase Cst-II, *N*-acetylgalactosaminyl-transferase CgtA, and galactosyltransferase CgtB. There is a strong association between the simultaneous presence of these genes and Guillain–Barré syndrome-associated *C. jejuni* strains. Knockout mutants of *C. jejuni* genes involved in lipo-oligosaccharide sialylation had reduced reactivity with anti-GM1 sera from Guillain–Barré syndrome patients, and did not induce an anti-GD1a IgG antibody response in mice. Lipo-oligosaccharide biosynthesis genes appear to be essential for the induction of anti-GM1 or anti-GD1a IgG antibody and the subsequent development of acute motor axonal neuropathy.

Summary

The concept that carbohydrate mimicry causes autoimmune disease provides a clue to the resolution of the pathogenesis of other immune-mediated diseases.

Keywords

acute motor axonal neuropathy, *Campylobacter jejuni*, ganglioside, Guillain–Barré syndrome, lipo-oligosaccharide, molecular mimicry

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Abbreviations

AIDP	acute inflammatory demyelinating polyneuropathy
AMAN	acute motor axonal neuropathy
GBS	Guillain–Barré syndrome

1350-7540

Introduction

Guillain–Barré syndrome (GBS), characterized by acute flaccid paralysis and areflexia, is the prototype of post-infectious autoimmune diseases. Most GBS patients have had gastrointestinal or upper respiratory symptoms one to two weeks before the onset of limb weakness. The relatively short period between the antecedent illness and neuropathy makes possible both the determination of the antecedent pathogen and its isolation from GBS patients. The Gram-negative bacterium, *Campylobacter jejuni*, a leading cause of acute gastroenteritis in humans, is the most frequent antecedent pathogen in GBS [1]. This review provides an update on molecular mimicry as a cause of GBS after *C. jejuni* infection.

A true case of molecular mimicry

Molecular mimicry is one mechanism by which infectious agents may trigger an immune response against autoantigens. Many study findings are consistent with the mimicry hypothesis, but none has convincingly demonstrated that mimicry is an important mechanism in the development of autoimmune disease in humans. Several examples of molecular mimicry between microbial and self components are known, but in most cases no epidemiological relationship between autoimmune disease and microbial infection has been established. Moreover, in some studies, no replica of human autoimmune disease has been obtained by immunization with the mimic of an infectious agent. Replicas associated with definite, epidemiological evidence of microbial infection are required to test the molecular mimicry theory of the development of autoimmune diseases.

Four criteria must be satisfied to conclude that a disease is triggered by molecular mimicry [2•]: (1) the establishment of an epidemiological association between the infectious agent and the immune-mediated disease; (2) the identification of T cells or antibodies directed against the patient's target antigens; (3) the identification

of microbial mimics of the target antigen; and (4) reproduction of the disease in an animal model. As reviewed here, GBS subsequent to *C. jejuni* enteritis fulfills all four criteria and provides the first verification that molecular mimicry is a cause of human autoimmune diseases.

Epidemiological association of *C. jejuni* with Guillain-Barré syndrome

An epidemiological study in England established the relationship between GBS and antecedent *C. jejuni* infection [3]. Serological assessments are useful for epidemiological studies, but there are neither standard methodologies as to which antigens to use nor standards of judgement. Because *C. jejuni* isolation is the standard for the diagnosis of this infection, epidemiological studies of a large number of *C. jejuni* isolates from GBS patients are required. Epidemiological features of more than 100 Japanese patients with GBS, from whom *C. jejuni* had been isolated, have been reported [4•]. *C. jejuni*-isolated GBS peaked in 10–30-year-old individuals, and the male : female ratio was 1.7 : 1. The dominance of young adult, male patients with *C. jejuni*-isolated GBS may be related to the preponderance of young adult and male patients who had had *C. jejuni* enteritis. The median latent period between antecedent symptoms and the onset of neuropathy was 10 days. Diarrhoea or abdominal pain preceded symptoms in 90% of those studied, and was often accompanied by fever.

On the basis of electrodiagnostic and pathological criteria, GBS is divided into acute inflammatory demyelinating polyneuropathy (AIDP) and acute motor axonal neuropathy (AMAN). Whereas electrodiagnostic studies have shown that *C. jejuni* infection is associated significantly with primary axonal dysfunction, the relationship between it and neurophysiology has been the subject of debate. To investigate whether *C. jejuni* infection does elicit AIDP, serial electrodiagnostic studies were conducted on *C. jejuni*-positive GBS patients [5•]. The presence of antecedent *C. jejuni* infection was determined by strict criteria; a positive *C. jejuni* serology and a history of diarrhoeal illness within the previous 3 weeks. Based on the electrodiagnostic criteria, 22 *C. jejuni*-positive patients were classified as having AMAN ($n = 16$, 73%), AIDP ($n = 5$, 23%), or were unclassified ($n = 1$) in the first studies. The five *C. jejuni*-positive patients with the AIDP pattern showed prolonged motor distal latencies but rapid normalization within 2 weeks. Eventually all showed the AMAN pattern. In contrast, patients with cytomegalovirus or Epstein–Barr virus-related AIDP had progressive increases in distal latencies during 8 weeks after onset. Patients with *C. jejuni*-related GBS had a transient slowing of nerve conduction that mimicked demyelination, but *C. jejuni* infection did not produce AIDP.

Autoantibodies against gangliosides in acute motor axonal neuropathy

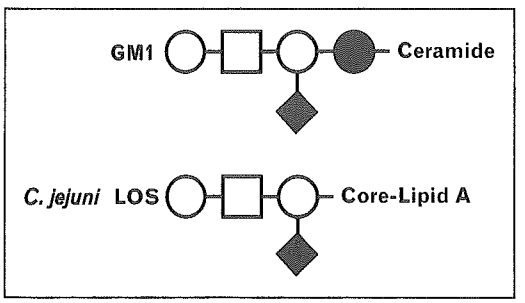
Autoantibodies against self-gangliosides are present in patients with GBS [6]. IgG antibodies to GM1 and GD1a are associated with AMAN or *C. jejuni* infection. Serial electrodiagnostic studies were performed on GBS patients who had IgG antibodies to either ganglioside [7]. Besides the simple axonal degeneration pattern, those patients showed transient conduction slowing/a block in distal or proximal nerve segments, mimicking demyelination, but their antiganglioside antibodies were not associated with AIDP.

C. jejuni mimic of gangliosides

Lipo-oligosaccharide is a major cell-surface structure in *C. jejuni* that is recognized by the host. A *C. jejuni* strain (CF90-26) isolated from an AMAN patient carrying anti-GM1 IgG antibody expressed an oligosaccharide structure [Gal β1–3 GalNAc β1–4 (NeuAc α2–3) Galβ], which protruded from the lipo-oligosaccharide core (Fig. 1) [8]. This terminal structure was identical to that of the terminal tetrasaccharide of the GM1 ganglioside. Another *C. jejuni* strain (16971.94GSH) isolated from a patient with GBS carried a GM1-like lipo-oligosaccharide, but neither antiganglioside antibodies nor electrodiagnosis was investigated [9]. A *C. jejuni* strain (ATCC43446) from an enteritis patient also had GM1 and GD1a-like lipo-oligosaccharides [10].

C. jejuni strains (OH4382 and OH4384) isolated from two patients with GBS, respectively, carried GD3 and GT1a-like lipo-oligosaccharides [10]. Both patients had external ophthalmoplegia, and one was comatose [11]. Overlapping GBS and Fisher syndrome and overlapping GBS and Bickerstaff's brainstem encephalitis, respectively, were diagnosed in these patients according to our diagnostic criteria [12]. Whether the patients carried anti-GQ1b IgG antibody was not tested, but inoculations of the lipo-oligosaccharides of both *C. jejuni* strains induced anti-GQ1b antibodies in mice [13].

Figure 1. Molecular mimicry of GM1 ganglioside and *Campylobacter jejuni* lipo-oligosaccharide



LOS, Lipo-oligosaccharide. ○ Galactose; ▣ *N*-Acetylgalactosamine; ◆ *N*-Acetylneuraminic acid; ● Glucose.

Animal models of acute motor axonal neuropathy

Gangliosides extracted from bovine brain tissue have been widely used in western Europe and South America as therapeutic agents for various neurological disorders. After receiving bovine brain ganglioside, some patients developed AMAN, and anti-GM1 IgG antibody was detected in them [14]. An AMAN model was established by sensitization of Japanese white rabbits with a bovine brain ganglioside mixture that included GM1 or with an isolated GM1 [15]. The rabbits developed high anti-GM1 IgG antibody titres, then flaccid limb weakness of acute onset with a monophasic course. Pathological findings in their peripheral nerves showed predominant Wallerian-like degeneration with neither lymphocytic infiltration nor demyelination. IgG was deposited on the axons of the ventral roots, internodal axolemmas, and nodes of Ranvier. Cauda equina and spinal nerve root specimens from the paralysed rabbits showed macrophage infiltration in the periaxonal space [16]. Surrounding myelin sheaths were almost intact. These findings correspond well with pathological findings for human AMAN [17,18]. This AMAN rabbit model was also reproducible in New Zealand white rabbits [19].

The most straightforward way to verify whether molecular mimicry between microbes and autoantigens causes GBS is to establish a GBS model by the immunization of animals with components of antecedent infectious agents. An AMAN model was established by the immunization of Japanese white rabbits with *C. jejuni* lipo-oligosaccharide bearing a GM1-like structure [20••]. On sensitization with this GM1-like lipo-oligosaccharide, rabbits developed high anti-GM1 IgG antibody titres and subsequent flaccid limb weakness. Their nerve roots had occasional macrophages in the periaxonal spaces surrounded by almost intact myelin sheaths. Axons of these nerve fibres showed various degrees of degeneration. Demyelination and remyelination were rare. These findings, compatible with the features of human AMAN, are evidence that rabbits inoculated with *C. jejuni* lipo-oligosaccharide constitute a valid AMAN model. This is the first definitive replica of a human autoimmune disease produced by immunization with the mimic of an infectious agent associated with epidemiological evidence of microbial infection.

Progress towards fulfilling the postulates of Witebsky *et al.*

Documentation of the autoimmune aetiology of a human disease is difficult. Witebsky *et al.* [21] developed standards for such documentation: (1) It should be possible to demonstrate circulating antibodies, active at body temperature in the serum of patients who have the disease, or cell-bound antibodies (in contemporary terms, this is cell-mediated immune reactivity); (2) The antigen against which the antibody is directed should be characterized or even isolated; (3) Antibodies should be produced against the same antigen in experimental animals; (4) Pathological changes that appear in the corresponding tissue from an actively sensitized animal should be similar or identical to those found in the human disease. Although a number of human diseases are believed to have an autoimmune aetiology or, at least, an autoimmune component, clearly only a few formally fulfil the criteria of Witebsky *et al.* [21]. Myasthenia gravis does, whereas other diseases such as multiple sclerosis do not.

As reported, GM1 is one of the autoantigens for IgG antibodies found in some patients with AMAN [6]. The IgG class of the autoantibody against GM1 was produced in experimental animals. Pathological changes that appeared in the peripheral nerves were identical to those in human AMAN [15,16]. More data are needed to satisfy the postulates of Witebsky *et al.* [21] involving the induction of clinical and pathological diseases by the passive transfer of anti-GM1 IgG antibody. This systemic transfer of anti-GM1 IgG antibody from AMAN patients did not induce paralysis in mice, but the autoantibody did block muscle action potentials in a rat muscle–spinal cord co-culture [20••].

Passive transfer attempted with systemically administered mouse anti-GD1a IgG antibody did not cause nerve fibre degeneration despite high circulating autoantibody titres [22••]. Half of a population of mice given an intraperitoneal implant of anti-GD1a IgG antibody-secreting hybridoma, however, developed a patchy, predominantly axonal neuropathy that affected a small number of nerve fibres. Mice implanted with the hybridoma had a leaky blood–nerve barrier compared with those that received systemically administered anti-GD1a IgG antibody. These findings suggest that in addition to circulating anti-ganglioside antibodies, such factors as antibody accessibility and nerve fibre resistance to antibody-mediated injury are important in the development of a neuropathy. *Ex vivo* nerve-muscle preparations from GD1a-overexpressing, GD3 synthase knockout mice were exposed to mouse anti-GD1a IgG antibody in the presence of a source of complement [23•]. Dense antibody and complement deposits were found only on presynaptic motor axons accompanied by severe ultrastructural damage and electrophysiological blockade of motor nerve terminal functions. Identical paralysing effects were observed on testing human anti-GD1a-positive sera.

C. jejuni genes associated with Guillain–Barré syndrome

An epidemiological study performed in Sweden showed that one of 3285 *C. jejuni* enteritis patients developed GBS [24]. Only a minority of those who have *C. jejuni* enteritis develop GBS, possibly because of both bacterial

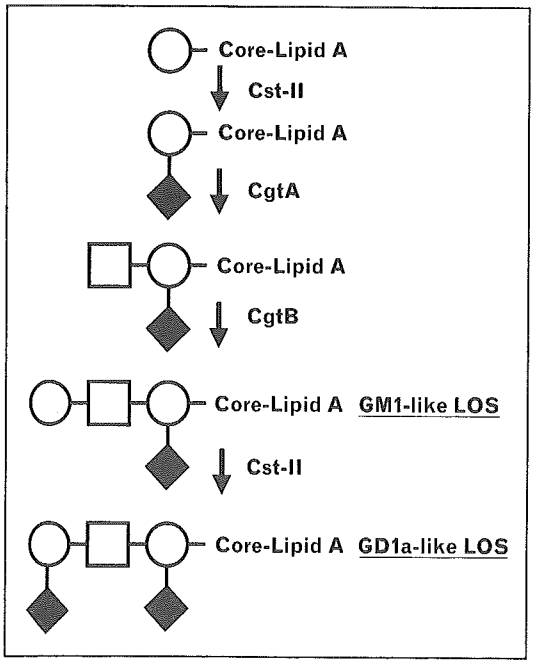
and host factors. Penner's serotyping revealed that in Japan and South Africa, respectively, HS:19 and HS:41 were more common in GBS isolates than in enteritis isolates [4•,25]. In contrast, no clustering of a specific serotype was found in GBS-related isolates in England and the Netherlands [3,26]. Previous studies failed to find a specific *C. jejuni* genotype for GBS [26,27].

As stated, ganglioside mimicry of *C. jejuni* lipo-oligosaccharide is a cause of GBS. Ganglioside-like lipo-oligosaccharide is synthesized by sialyltransferase Cst-II, *N*-acetylgalactosaminyl-transferase CgtA, and galactosyltransferase CgtB (Fig. 2) [28]. Compared with gastroenteritis-related isolates, GBS-related *C. jejuni* isolates have a strong association with the expression of GD1a mimicry [29]. The presence of some genes (*cst-II*, *cgtA*, and *cgtB*) involved in ganglioside mimicry is also associated with GBS-related strains. These lipo-oligosaccharide biosynthesis genes cluster at the lipo-oligosaccharide biosynthesis gene locus [30]. A specific type of gene locus, which includes these three genes, is associated with GBS and with the GM1-like lipo-oligosaccharide [31••].

The *cst-II* gene encodes an enzyme that transfers sialic acid to the lipo-oligosaccharide and another gene, possibly an enzyme, which synthesizes sialic acid [28]. Because both genes are involved in lipo-oligosaccharide sialylation, they are essential for ganglioside-like lipo-oligosaccharide synthesis. Mutants of *C. jejuni* that lack these genes have been made and analysed [31••]. Mass spectrometry analysis identified a mixture of GM1 and GD1a-like structures in wild-type *C. jejuni* strains isolated from GBS patients. The mutants expressed such non-sialylated structures as asialo-GM3, asialo-GM2, and asialo-GM1-like lipo-oligosaccharides. The knockout mutants, unlike the wild types, had decreased reactivity to the sera of GBS patients. GM2/GD2 synthase knockout mice, which lack GM1 and GD1a, are immune-naive hosts that can be used to obtain high-titre antiganglioside antibody responses. Immunization with the wild-type strain induced an anti-GD1a IgG antibody response in these mice, whereas immuniztion with the mutant strains did not. This shows that the genes involved in lipo-oligosaccharide sialylation are essential for the induction of antiganglioside antibodies.

In contrast, no host susceptibility genes associated with the development of GBS have yet been identified. Lipopolysaccharide receptors CD14 and Toll-like receptor 4 are important in antigen presentation and intracellular signaling, but the functional polymorphisms in *CD14* and *TLR4* are not associated with susceptibility to *C. jejuni*-associated GBS [32]. Fas polymorphisms are associated with the presence of antiganglioside antibodies in GBS [33].

Figure 2. Enzymatic synthesis of GM1 and GD1a-like lipo-oligosaccharides



○ Galactose; □ *N*-Acetylgalactosamine; ◆ *N*-Acetylneuraminic acid. LOS, Lipo-oligosaccharide.

Pathogenesis of acute motor axonal neuropathy subsequent to *C. jejuni* enteritis

C. jejuni, which simultaneously carries the lipo-oligosaccharide biosynthesis genes *cst-II*, *cgt-A*, and *cgt-B*, may express GM1 or GD1a-like lipo-oligosaccharide on its cell surfaces. Infection by such *C. jejuni* strains could induce anti-GM1 or anti-GD1a IgG production in patients who have certain immunogenetic backgrounds. Anti-GM1 or anti-GD1a IgG antibody would bind to GM1 or GD1a at the nodes of Ranvier. Complements would be deposited, and the nodes would lengthen. Macrophages would be recruited to some nodes as a result of complement activation. These local events at the nodes would disrupt the junctional complexes between the axolemma and myelin terminal loops at the paranodes. As a result, IgG and complement would enter the periaxonal space at the internodes. Complement activation within the periaxonal space would recruit macrophages. Complements with and without macrophages would then induce axonal degeneration of the motor fibres.

Conclusion

Convincing evidence has shown that molecular mimicry between GM1 and *C. jejuni* is a cause of AMAN. Bacterial lipo-oligosaccharide biosynthesis genes appear to be essential for the production of antiganglioside antibodies

and the subsequent development of AMAN. The susceptibility genes of the host have now to be clarified.

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Electrophoresis-assisted open-tubular liquid chromatography/mass spectrometry for the analysis of lipooligosaccharide expressed by *Campylobacter jejuni*

Lipooligosaccharide (LOS) is the major component of the external membrane of *Campylobacter jejuni*. LOS contains a hydrophobic moiety, lipid A, and a hydrophilic moiety, oligosaccharide. Due to the unique mimicry of human ganglioside structures and potential involvement in the induction of the autoimmune polyneuropathies, Guillain–Barré and Miller Fisher syndromes, the structural characterization of *C. jejuni* LOS has received much attention. We have been using capillary zone electrophoresis–mass spectrometry to analyze *O*-deacylated LOS from *C. jejuni*. In an attempt to optimize the separation conditions, the effect of methanol on the separation of LOS was investigated. It was found that methanol resulted in stronger adsorption of LOS onto the wall of fused-silica capillary. In this paper, we applied this adsorption to perform electrophoresis-assisted open-tubular liquid chromatography electrospray mass spectrometry for the analysis of *O*-deacylated LOS mixtures from *C. jejuni*. The analytical potential of the proposed strategy for the analysis of *O*-deacylated LOS glycoforms from five bacterial colonies is demonstrated. Online tandem mass spectrometry is shown to provide a powerful tool for characterization of variations in the hexosamine backbone, phosphorylation of the lipid A, and sialic acid sequence information.

Keywords: *Campylobacter jejuni* / Capillary electrophoresis / Lipooligosaccharide / Mass spectrometry / Open-tubular liquid chromatography DOI 10.1002/elps.200500145

1 Introduction

Campylobacter jejuni, a Gram-negative bacterium, has become the leading cause of bacterial gastroenteritis worldwide [1, 2]. The lipooligosaccharide (LOS) plays an important role in its pathogenesis, due to their unique mimicry of human ganglioside structures, which can potentially lead to the induction of autoimmune polyneuropathies, Guillain–Barré (GBS) and Miller Fisher syndromes [3–6]. Generally, LOS consists of a hydrophilic oligosaccharide covalently coupled to a hydrophobic lipid portion, *i.e.*, lipid A. As shown in Fig. 1, a structural model consisting of a conserved di-L-*glycero*-D-*manno*-heptosyl inner-core moiety in which heptose residues can provide a point for elongation by carbohydrate mimicking of human ganglioside structures or for attachment of non-carbohydrate substitutes is now well-established [7, 8]. For most Gram-negative bacteria, such as *Haemophilus influenzae* and *Neisseria meningitidis*, the structure of lipid A has been found to consist of a 1,6-linked D-glucosamine disaccharide substituted by *N*-linked 3-hydroxytetradecanoic acid at C-2 and C-2' and phosphomonoester groups at C-1 and C-4' [9–13]. However, the lipid A backbone of *C. jejuni* was found to consist of three (1–6)-linked hexosamine disaccharides: a D-glucosamine (2-amino-2-deoxy-D-glucose, or GlcN) disaccharide β-D-glucosaminyl-(1–6)-D-glucosamine], a hybrid disaccharide of 2,3-diamino-2,3-dideoxy-D-glucose (GlcN3N), and D-glucosamine, and a 2,3-diamino-2,3-dideoxy-D-glucose disaccharide, alternatively [14, 15]. The lipid A can carry up to six molecules of ester- and amide-bound fatty acids. Significant heterogeneity is observed with one or both glucosamine residues being replaced with the sugar GlcN3N [7, 8, 14, 15].

Considering the importance of carbohydrate moieties in the mechanism of host mimicry, there is a great need to develop rapid and sensitive methods for microscale

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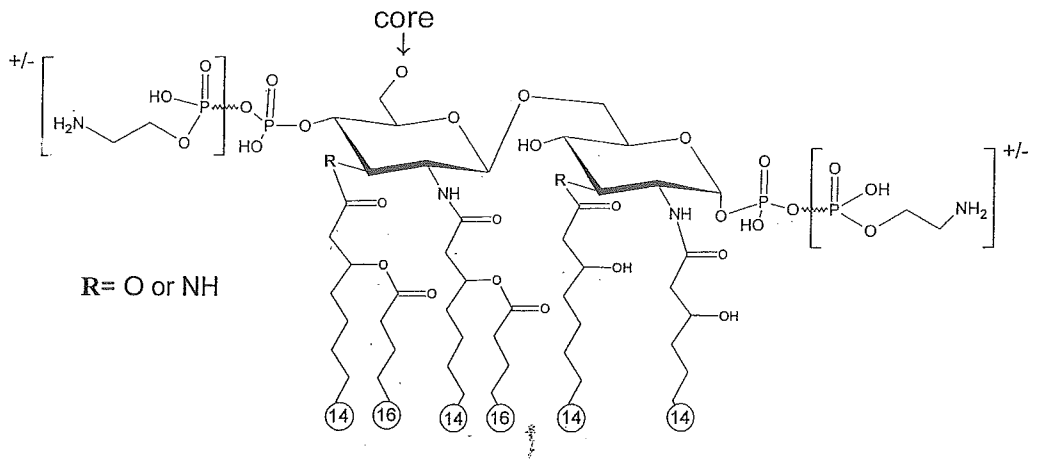
Abbreviations: EA-OTLC, electrophoresis-assisted open-tubular liquid chromatography; **GlcN**, 2-amino-2-deoxy-d-glucose; **GlcN3N**, 2,3-diamino-2,3-dideoxy-D-glucose; **Hex**, hexose; **IPA**, isopropanol; **KDO**, 3-deoxy-D-*manno*-oct-2-ulosonic acid; **LOS**, lipooligosaccharide; **Neu5Ac**, *N*-acetylneuraminic acid; ***P***, phosphate; ***P*Etn**, phosphoethanolamine; ***PP*Etn**, pyrophosphoethanolamine



$(Neu5Ac)_{1-4}$ —Gal —Hep — Hep — KDO — Lipid A
Glc KDO

Structure A

Gal—GalNAc— Gal — Hep — Hep — KDO — Lipid A
$(Neu5Ac)_2$ Neu5Ac Glc KDO

Structure B

Hex_{5-7} $HexNAc_1$ $Neu5Ac_1$ Hep_2 KDO_2 Lipid A

Structure C

Figure 1. Structure of the lipid A and LOS-core of *C. jejuni* strain GC149. LOS-core structures A and B are proposed based on similarities with *C. jejuni* strains OH4382 and OH4384, respectively [7].

analysis of complex *C. jejuni* LOS. Capillary electrophoresis (CE) is a high-resolution technique for the separation of complex biological mixtures and has been widely applied to biological analysis. We have been using capillary zone electrophoresis–mass spectrometry (CZE-MS) method to analyze trace levels of *O*-deacylated lipopolysaccharide (LPS) samples of *H. influenzae* and *N. meningitidis* [16–19]. In those applications, anhydrous hydrazine treatment under mild conditions (37°C, 1 h) was employed to remove the *O*-linked fatty acids leaving only *N*-linked fatty acid attached to each glucosamine. Hydrazinolysis along with the enzyme treatment provide a method to the sample impurity and most importantly, improve the solubility and reduce the aggregation of LPS in aqueous solutions.

Based on the chemical structure, the *O*-deacylated lipid A of *C. jejuni* can contain up to four *N*-linked fatty acids in the GlcN3N-GlcN3N disaccharide, when labeled "R" (Fig. 1) are present as amides (NH). Structures A and B are proposed based on similarities with *C. jejuni* strains OH4382 and OH4384, respectively [7]. It must be pointed out that the structural model for LOS-core C has not been established, although similar linkages for 3-deoxy-D-*manno*-oct-2-ulosonic acid (KDO) and L-*glycero*-D-*manno*-heptose (Hep) residues are expected. Nevertheless, presence of two additional fatty acid chains significantly increases the adsorption of LOS onto the fused-silica capillary surface. Usually, this adsorption interferes with CZE separation and results in problems, such as peak tailing, unstable electroosmotic flow (EOF), loss of separation efficiency, *etc.* To

optimize the separation conditions, the effect of methanol on the CZE separation of LOS was investigated. It was found that methanol resulted in stronger adsorption of LOS onto the wall of fused-silica capillary. For example, when 100% methanol with 50 mM ammonium acetate was used as background electrolyte, no LOS was detected within 8 min. The ability of methanol to enhance the adsorption of LOS was exploited and applied to capillary open-tubular chromatography assisted by electrophoresis. In recent years, applications of open-tubular capillary liquid chromatography (OT-CLC) and capillary electrochromatography (OT-CEC) have become an important tool in separation science [20–24]. It is believed that the protocol based on the adsorption effect of capillary wall provides a promising approach for the analysis of the trace level LOS.

2 Materials and methods

2.1 Materials

Fused-silica capillaries with 185 μm OD × 50 μm ID were purchased from Polymicro Technologies (Phoenix, AZ, USA). Methanol and isopropanol (IPA) were from EM Science (Gibbstown, NJ, USA). Anhydrous hydrazine and ammonium acetate were purchased from Fisher Scientific (Fair Lawn, NJ, USA) and formic acid from BDH (Toronto, Canada). The enzymes, proteinase K, deoxyribonuclease I (DNase) and ribonuclease (RNase) were purchased from Sigma (St. Louis, MO, USA).

2.2 Samples

2.2.1 Growth conditions and sample preparation

C. jejuni strain GC149 (HS:31; LOS class A; *cst-II* [Asn 51] genotype) is a clinical isolate from a 14-year-old boy who suffered from Miller Fisher syndrome [25]. *C. jejuni* was grown for approximately 18 h on Mueller-Hinton agar (Difco, Detroit, MI, USA) under microaerophilic conditions at 37°C. The cells from half a plate or from 5, 10, or 50 colonies were resuspended in 0.3 mL of PBS, pH 7.4. To this, 1.0 mL of 100% ethanol was added to give a 70% ethanol final concentration. The cells were incubated at room temperature for 1 h, pelleted and washed twice with 1.0 mL of 100% ethanol followed by two washes with 1.0 mL of acetone. The sample was then allowed to air-dry.

2.2.2 Preparation of *O*-deacylated LOS

The dried cells were resuspended in 200 μL of deionized water containing 60 μg/mL proteinase K and incubated at 37°C for 4 h. The temperature was raised to 75°C for 10 min to stop the digestion and the sample was lyophilized. The cells were resuspended in 200 μL of 20 mM ammonium acetate buffer (pH 7.5) containing 200 μg/mL RNase and 100 μg/mL DNase, and incubated at 37°C for 6 h before being lyophilized. The cell digests were then stirred with 200 μL of hydrazine at 37°C for 2 h to cleave *O*-linked fatty acids of the LOS. The sample was placed in an ice bath and the excess hydrazine was destroyed with cold acetone in dry ice (6 × 200 μL). The *O*-deacylated LOS was recovered in a pellet after centrifugation at 16 000 × *g* for 15 min. The pellet was then washed once with 400 μL acetone and recovered again by centrifugation. The washed pellet was resuspended in water and centrifuged at 16 000 × *g* for 15 min. The LOS-containing supernatant was recovered and lyophilized.

2.3 Methods

2.3.1 Infusion electrospray ionization (ESI)-MS

In this study, a sheathflow interface was used and the sheath solution of IPA/methanol mixture (2:1) was applied using a syringe pump with a flow rate of 1.0 μL/min. The sample was delivered using a Prince CE system (Prince Technologies, Emmen, The Netherlands) at 500 mbar with a bare fused-silica capillary column (90 cm length, 185 μm OD × 50 μm ID) as a transfer line, which corresponds to a flow rate of *ca.* 0.5 μL/min. The ESI voltage applied on the sprayer was set at −5.2 kV in the negative ion detection mode. Data acquisition was performed in the range of *m/z* 600–2000 at a scan rate of 3 s/spectrum.

2.3.2 CZE-MS

CE was performed using a Prince CE system. All aqueous solutions were filtered through a 0.45 μm filter (Millipore, Bedford, MA, USA) before use. All CZE-MS experiments were performed as described previously in detail [17]. The separation was obtained on about 90 cm length bare fused-silica capillary (185 μm OD × 50 μm ID). The CE system was coupled to a QSTAR mass spectrometer (Applied Biosystems/Sciex, Concord, Canada) *via* a microspray interface. A sheath solution (isopropanol–methanol, 2:1) was delivered at a flow rate of 1.0 μL/min to a low dead volume tee (250 μm ID, Chromatographic Specialties, Brockville, Canada). In all of the CZE-MS experiments, 60 nL of sample was typically injected by using 300 mbar for duration of 0.2 min. Separations were obtained using 30 mM morpholine in deionized water, pH 9.0. A separation voltage of 20 kV, together with a pressure of 500 mbar, was used for the fast CZE-MS analysis. The ESI voltage applied on the sprayer was set at −5.2 kV in the negative ion detection mode. Data acquisition was performed in the range of *m/z* 600–2000 at a scan rate of 3 s/spectrum.

2.3.3 Electrophoresis-assisted (EA)-OTLC-MS

A large volume (typically 1.0 μL) of a sample was injected, followed by washing the capillary column with 1.0 μL 100% methanol. A small plug of 1.0 M ammonium acetate in deionized water (60 nL) was injected to elute the adsorbed *O*-deacylated LOS from capillary surface. The separation was performed using 30 mM morpholine, pH 9.0. A separation voltage of 20 kV, together with a pressure of 500 mbar was applied for the EA-OTLC-MS analysis. The ESI voltage applied on the sprayer was set at −5.2 kV in the negative ion detection mode. Data acquisition was performed in the range of *m/z* 600–2000 at a scan rate of 3 s/spectrum. Nitrogen was used as the collision gas at a recorded pressure of 7.0×10^{-5} Torr (1 Torr = 133.3 Pa). Unless specified, tandem mass spectra were obtained at collision energy of 120 eV (laboratory frame of reference). Fragment ions formed in RF-only quadrupole were recorded using the time of flight mass analyzer.

3 Results and discussion

3.1 Direct infusion ESI-MS

Generally, direct infusion ESI-MS is the easiest way to develop a high-throughput technology. However, this technique does not always work for the analysis of complex biological samples, especially for those samples with a high content of salts. Results of the direct infusion ESI-MS analysis of *O*-deacylated LOS from *C. jejuni* strain GC149 are presented in Fig. 2. As expected, the total ion chromatogram (TIC) indicated that the sample reached the electrosprayer in about 3 min. However, the extracted mass spectra did not indicate any ions relating to the compound of interest. Although the adsorption of LOS onto the capillary surface is an important factor, the signal depress effect in electrospray may also be responsible for this observation. For analysis of LOS, it is essential to remove salts and other low-molecular weight impurities (*e.g.*, amino acids and nucleotide sugars). To this end, we attempted to perform online desalting by coupling a CE system.

3.2 CZE-MS

CZE-MS offers a high sensitivity technology for characterization of LPS expressed by *H. influenzae* and *N. meningitidis* [16–19]. Our previous studies demonstrated that similar strategy could be applied to the direct analysis of *C. jejuni* LOS from 10^8 to 10^{10} cells [6, 8]. In the CZE separation, a key factor for reproducible results is the reproducibility of EOF. Due to a high content of salts and

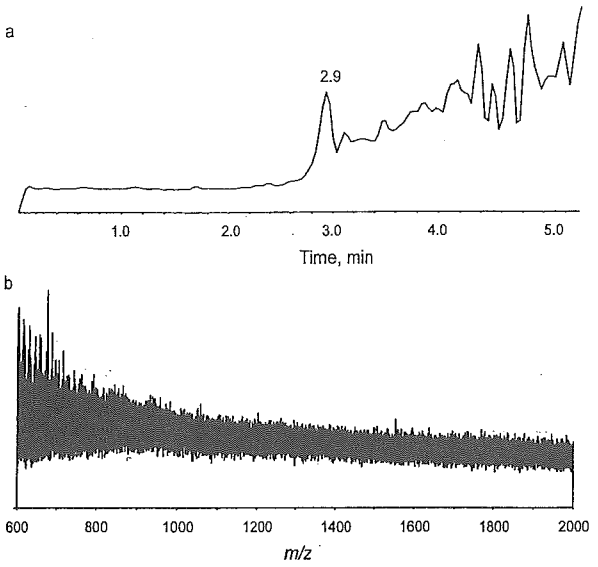


Figure 2. Direct infusion ESI-MS analysis of the *O*-deacylated LOS from *C. jejuni* strain GC149. A sheathflow interface was used and the sheath solution of IPA/methanol mixture (2:1) was delivered with a flow rate of 1.0 μL/min. Data were acquired in negative detection mode with electrospay voltage of −5.2 kV. Sample was delivered using 500 mbar, which corresponds to a flow rate of *ca* 0.5 μL/min. (a) Total ion chromatogram (TIC) (*m/z* 600–2000). (b) Extracted mass spectra from 2.9 to 5.0 min (average of 42 scans).

other contaminants (amino acids, nucleotide sugars, or lipids) in the samples, the reproducibility of EOF is relatively very poor for analysis of LOS from cell cultures. To minimize the effect of highly nonreproducible EOF on the application of this technique, an assist pressure of 500 mbar was applied during the CZE separation. This pressure resulted in a flow rate of no less than 0.5 μL/min and provided a fast separation of LOS from salts and other major components as shown in Fig. 3, in which the same *O*-deacylated LOS sample was used. The triply charged ions at *m/z* 1009.9, 1050.9, 1125.9, and 1148.0 (Fig. 3b), together with corresponding quadruply charged counterparts, indicated that the major glycoforms contained two ($Neu5Ac_2$) and three ($Neu5Ac_3$) sialic acid residues, respectively. The parallel series of triply charged ions displaced by 41 *m/z* units could be attributed to a subpopulation of glycoforms containing an additional phosphoethanolamine (*P*Etn) residue in the lipid A region. Another parallel series of triply charged ions displaced by 75 *m/z* units corresponded to an additional C14:0 (3-OH) *N*-linked fatty acid chain. As summarized in Table 1, heterogeneity of both the oligosaccharide and lipid A structures has led to an extensive repertoire of glycoforms. These observations suggested that the CZE-MS method for the analysis of *O*-deacylated LOS prevents the