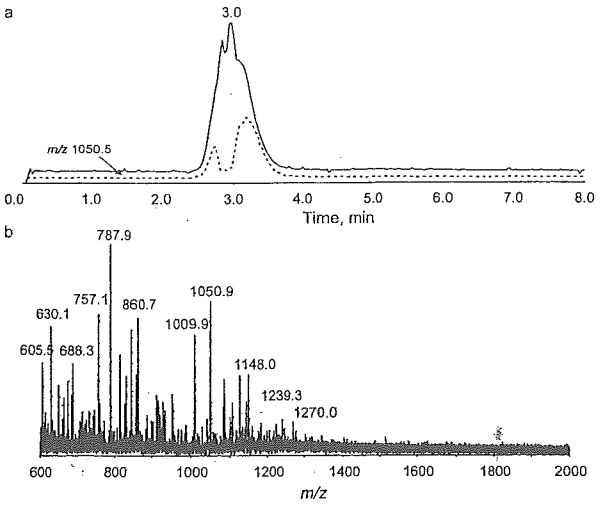


Figure 3. CZE-MS analysis of the *O*-deacylated LOS from *C. jejuni* strain GC149. (a) TIE (*m/z* 600–2000). (b) Extracted mass spectra from 3.2 to 3.5 min (average spectra of six scans). Separation conditions: bare fused-silica (90 cm × 50 μm ID, 190 μm OD), 30 mM morpholine, pH 9.0, +20 kV, 500 mbar. Data were acquired in negative detection mode with electrospay voltage of −5.2 kV.

destruction of important constituents that are acid sensitive, such as sialic acid, which could be lost during the mild acid hydrolysis routinely used to isolate LOS core. Variations in the hexosamine backbone and in phosphorylation of the lipid A structure could easily be detected. However, the adsorption of LOS on the capillary surface, associated with the greater hydrophobility of lipid A portion, prevented further improvement in the sensitivity.

3.3 EA-OTLC-MS and EA-OTLC-MS/MS

The unique chemical structure of *O*-deacylated LOS expressed by *C. jejuni* causes its strong adsorption onto the wall of the fused-silica capillary and makes it difficult to analyze *C. jejuni* LOS from small colony samples. In order to overcome this adsorption problem, the effect of methanol on the CZE performance was investigated. As shown in Fig. 4, different amounts of methanol in 50 mM ammonium acetate buffers were evaluated, with presence of 0, 25, 50, 75, and 100% methanol, respectively. Using the 50 mM ammonium acetate buffer without addition of methanol, the reconstructed ion electropherogram

Table 1. MS data and proposed composition of the *O*-deacylated LOS from *C. jejuni* strain GC149[a)]

Lipid A	Neu5Ac	Observed ions ($[M - 3H]^{3-}$)	Observed mass (Da)	Calculated mass (Da)	Core structure
GlcN3N-GlcN-(*P*, *PP*Etn)	1	912.2	2739.6	2740.1	A (Hex2)
GlcN3N-GlcN-(*PP*Etn, *PP*Etn)	1	953.5	2863.5	2863.1	A (Hex2)
GlcN3N-GlcN-(*P*, *PP*Etn)	2	1009.5	3031.5	3031.2	A (Hex2)
GlcN3N-GlcN-(*PP*Etn, *PP*Etn)	2	1050.6	3154.8	3154.2	A (Hex2)
GlcN3N-GlcN3N-(*P*, *PP*Etn)	2	1084.6	3256.8	3256.4	A (Hex2)
GlcN3N-GlcN-(*P*, *PP*Etn)	3	1106.6	3322.8	3322.3	A (Hex2)
GlcN3N-GlcN-(*PP*Etn, *PP*Etn)	3	1147.6	3445.8	3445.3	A (Hex2)
GlcN3N-GlcN3N-(*PP*Etn, *PP*Etn)	3	1222.7	3671.1	3670.5	A (Hex2)
GlcN3N-GlcN-(*P*, *PP*Etn)	4	1203.7	3614.1	3613.4	A (Hex2)
GlcN3N-GlcN-(*PP*Etn, *PP*Etn)	4	1244.7	3737.1	3736.4	A (Hex2)
GlcN3N-GlcN-(*P*, *PP*Etn)	3	1228.4	3688.2	3687.4	B (Hex3)
GlcN3N-GlcN-(*PP*Etn, *PP*Etn)	3	1269.4	3811.2	3810.4	B (Hex3)
GlcN3N-GlcN3N-(*PP*Etn, *PP*Etn)	3	1344.4	4036.2	4035.6	B (Hex3)
GlcN3N-GlcN-(*P*, *P*)	1	1100.6	3304.8	3306.4	C (Hex5)
GlcN3N-GlcN-(*P*, *PP*Etn)	2	1238.7	3719.1	3720.4	C (Hex5)
GlcN3N-GlcN3N-(*P*, *P*)	3	1369.3	4110.9	4113.7	C (Hex5)
GlcN3N-GlcN-(*P*, *PP*)	1	1181.6	3547.8	3548.3	C (Hex6)
GlcN3N-GlcN3N-(*P*, *P*)	1	1230.4	3694.2	3693.6	C (Hex6)[b)]
GlcN3N-GlcN-(*P*, *PP*Etn)	1	1196.3	3591.9	3591.4	C (Hex6)[b)]
GlcN3N-GlcN3N-(*P*, *PP*Etn)	1	1271.4	3817.2	3816.7	C (Hex6)[b)]
GlcN3N-GlcN-(*P*, *PP*Etn)	1	1250.4	3754.2	3753.4	C (Hex7)[b)]
GlcN3N-GlcN3N-(*P*, *PP*Etn)	1	1325.0	3978.0	3978.6	C (Hex7)[b)]

a) Monoisotope mass units were used for calculation of molecular mass values based on proposed compositions as follows: Hex (galactose and glucose), 162.05; Hep, 192.06; GlcN, 161.07; GlcN3N, 160.08; *N*-acetylgalactosamine, 203.08; KDO, 220.06; Neu5Ac, 291.10; *P*, 79.97; *P*Etn, 123.01; 3-OH C14:0, 226.19; H_2O, 18.01. *PP*Etn, pyrophosphoethanolamine

b) Major glycoforms found in five colony and ten colony samples

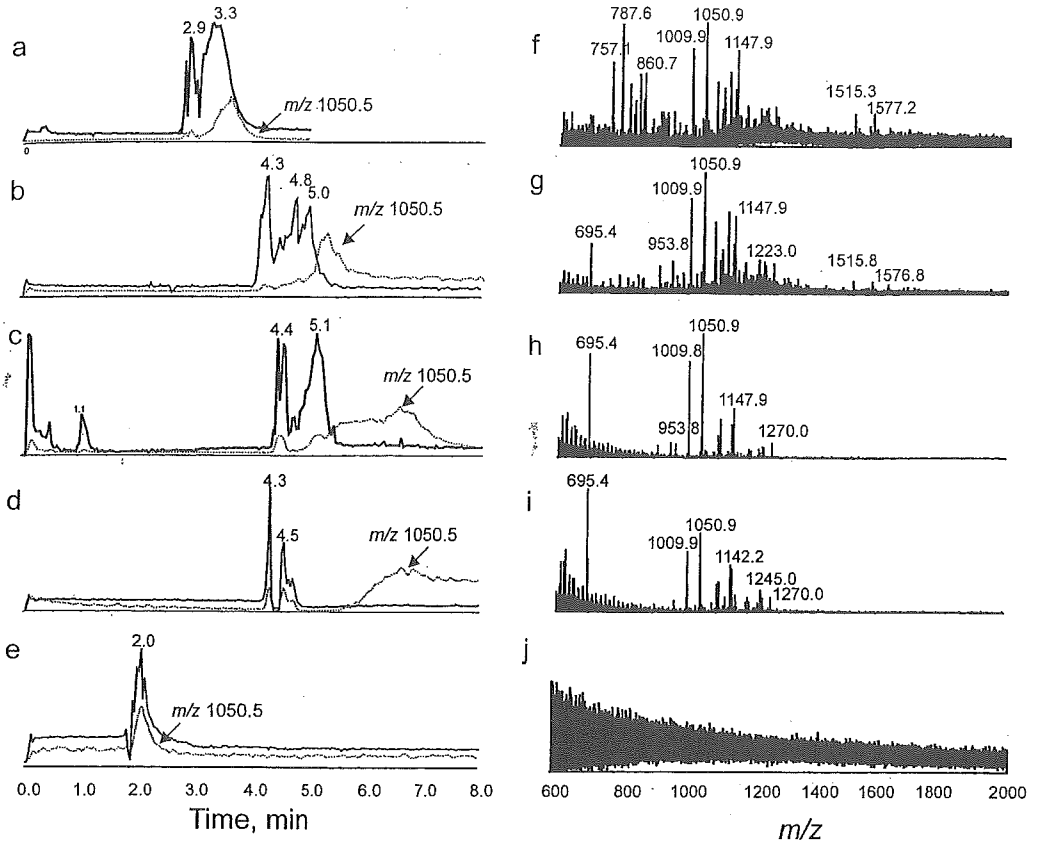


Figure 4. CZE-MS analysis of the *O*-deacylated LOS from *C. jejuni* strain GC149. (a)–(e) TIE (*m/z* 600–2000): (a) 50 mM ammonium acetate in 0% methanol; (b) 50 mM ammonium acetate in 25% methanol; (c) 50 mM ammonium acetate in 50% methanol; (d) 50 mM ammonium acetate in 75% methanol; (e) 50 mM ammonium acetate in 100% methanol. (f)–(j) Extracted mass spectra: (f) average spectra of six scans (3.5–3.8 min); (g) average spectra of six scans (5.1–5.4 min); (h) average spectra of 20 scans (5.5–6.5 min); (i) average spectra of 40 scans (6.0–8.0 min); (j) average spectra of eight scans (1.8–2.2 min).

(RIE) showed two peaks at *m/z* 1050.5 (Fig. 4a). The migration time was significantly increased with the increase in the methanol content. The peak width was also broadened as a result of a higher amount of methanol being added to the CZE background electrolyte. When 100% methanol was used as separation "buffer" (containing 50 mM ammonium acetate), no LOS ions were detected within 8 min. Moreover, the extracted mass spectra have demonstrated that the quality of mass spectra in Figs. 4h and i was much better than that in Figs. 4f and g. To take advantage of this adsorption, we have examined whether an EA-OTLC-MS protocol could be developed. A large volume of a sample was loaded into the capillary and followed by a plug of pure methanol to enhance the adsorption. Then a small plug of 1.0 M ammonium acetate was injected to elute LOS from the capillary surface. Similar to chromatography mechanism, the ammonium ions are able to increase the solubility of LOS. A buffer of 30 mM morpholine, pH 9.0, was followed to further improve the sensitivity though sample stacking. The result of this EA-OTLC-MS analysis of *O*-deacylated LOS from *C. jejuni* strain GC149 is presented in Fig. 5. For this analysis, 1.0 μL of tenfold dilution of the sample used in Fig. 4 was injected. The total electrophoregram (TIE) together with RIE at *m/z* 1005.5 shows the separation of *O*-deacylated LOS from other components in the sample, with migration times of 2.1, 2.3, and 2.7 min, respectively. This corresponds to a tenfold improvement in concentration detection limits compared to that observed in previous CZE-MS (Fig. 4). To further examine the application to LOS structural characterization, EA-OTLC-MS/MS experiments were conducted for the same sample. Figures 6a–c present the tandem mass spectra at *m/z* 860.4, corresponding to a quadruply charged glycoform with trisialic acids, with different collision energies. Obviously the optimal collision energy is 30 V (or labora-

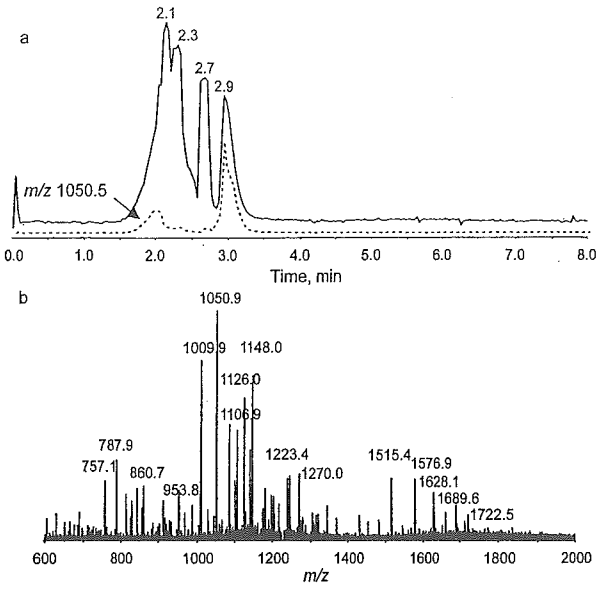


Figure 5. EA-OTLC-MS analysis of the *O*-deacylated LOS from *C. jejuni* strain GC149. One microliter of the tenfold dilution of the sample that was used in Fig. 3 was loaded. (a) TIE (*m/z* 600–2000). (b) Extracted spectra from 2.9 to 3.2 min (average spectra of six scans).

tory frame reference of 120 eV), in which predominant ions at *m/z* 290.1, 581.1, and 710.7 were observed. The doubly charged fragment ion at *m/z* 710.7 revealed that the lipid A portion is composed of a disaccharide (GlcN, GlcN3N), to which two pyrophosphoethanolamine (*PP*Etn) residues and three C14:0 (3-OH) fatty acids are linked. However, only minor peak at *m/z* 872.2 was detected, which gave no evidence of the existence of trisialic acid (Neu5Ac-Neu5Ac-Neu5Ac). Instead, it might lead to suggest that the existence of a monosialic acid and a disialic acid residue is attached to different sugar residues. In other words, the tandem mass spectra at improper charge state ions could lead to misidentification of the LOS structure for glycoforms containing three tandem sialic acid residues. An alternative strategy to increase the yield of sequence-specific fragment ions by collision-induced dissociation is to choose a lower charge state precursor ion. Using $[M - 3H]^{3-}$ at *m/z* 1147.6 as a precursor ion (Figs. 6d–f), MS/MS productions are observed at *m/z* 290.1 corresponding to single sialic acid residue, *m/z* 581.1 corresponding to disialic acid and *m/z* 872.2 corresponding to the trisialic acid fragment. The optimal collision energy was found to be 40 V or 120 eV (a

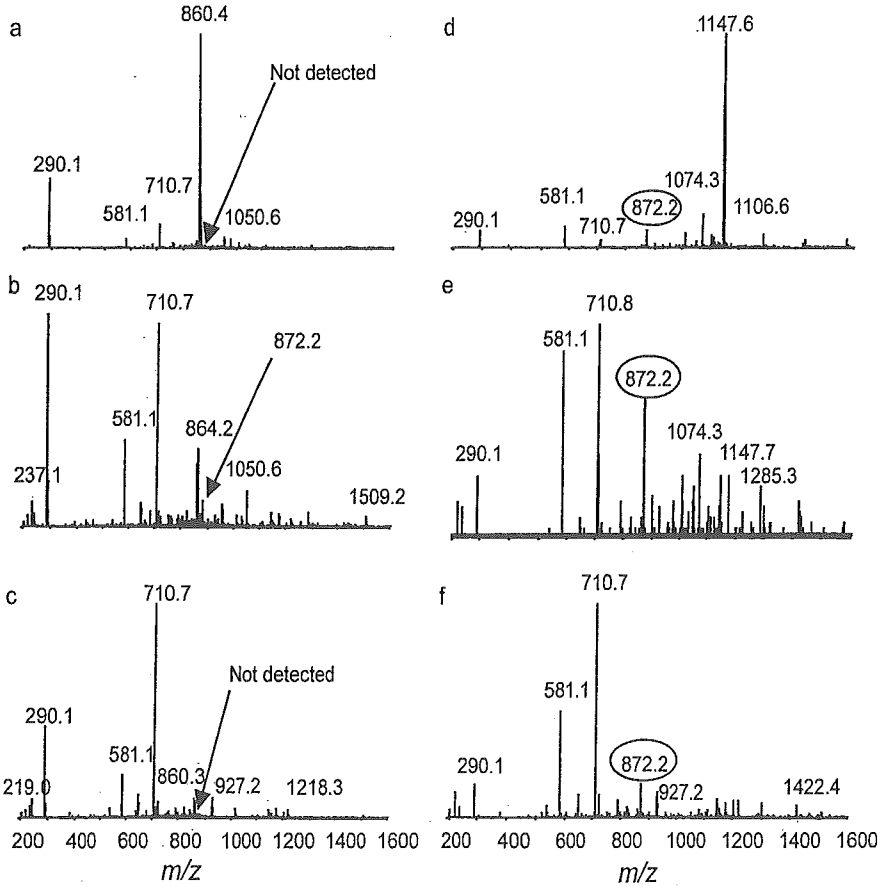


Figure 6. EA-OTLC-MS/MS analysis of the *O*-deacylated LOS from *C. jejuni* strain GC149 (average spectra of six scans). (a) MS/MS of quadruply charged ion at *m/z* 860.4, E_{lab} 80 eV; (b) MS/MS of triply charged ion at *m/z* 860.4, E_{lab} 120 eV; (c) MS/MS of quadruply charged ion at *m/z* 860.4, E_{lab} 160 eV; (d) MS/MS of triply charged ion at *m/z* 1147.6, E_{lab} 90 eV; (e) MS/MS of triply charged ion at *m/z* 1147.6, E_{lab} 120 eV; (f) MS/MS of triply charged ion at *m/z* 1147.6, E_{lab} 150 eV. Collision energy is laboratory frame reference.

laboratory frame reference). Nevertheless, trisialic acid fragments were also observed for the MS/MS spectra with lower (Fig. 6d) or higher energy (Fig. 6f). Localization of a negative charge at a particular site on the LOS profoundly affects the nature of fragment ions observed in low-collision energy tandem MS. Typically, the lipid A portion carries two negative charges (each on phosphate (*P*) or *PP*Etn residue), promoting formation of doubly charged fragment ions of lipid A (*m/z* 710.7). Therefore, it is not surprising that when the fragment containing two or more deprotonated sialic acid is generated, it will undergo further fragmentation to produce singly charged ions at *m/z* 290.1 (Fig. 6b).

3.4 EA-OTLC-MS and EA-OTLC-MS/MS for analysis of small colony samples

The EA-OTLC-MS and EA-OTLC-MS/MS technique for the analysis of trace level LOS was further demonstrated for bacterial isolates taken directly from plate cell cultures of *C. jejuni* strain GC149. The proposed strategy was evaluated using isolates in groups of 5, 10, and 50 colonies. The EA-OTLC-MS analysis of *O*-deacylated LOS samples obtained from 5, 10, and 50 colonies of *C. jejuni* strain GC149 is shown in Fig. 7. The observed ions in the 50-colony sample were similar to those detected for the whole plate cell cultures or its dilutions (Figs. 3b, 5b). The assignments of the detected ions are listed in Table 1. Surprisingly, the major glycoforms obtained from five and ten colonies were different from that of 50 colonies or whole plate cell cultures. To identify the composition of these unexpected ions, tandem mass spectra were obtained for the precursor at *m/z* 1272.0 (triply charged ion). As shown in Fig. 8, the fragment pattern is very similar to that of precursor ion at *m/z* 1147.6. For example, the observed fragment ion at *m/z* 290.1 indicated the presence of a single sialic acid residue. The predominant ion at *m/z* 762.1 (doubly charged ion) revealed that the lipid A portion of the molecule consisted of a disaccharide (GlcN3N, GlcN3N), to which one *P*, one *PP*Etn, and four *N*-linked fatty acid chains were attached. The fragment ion at *m/z* 1145.1 arose from loss of the lipid A moiety from the LOS molecule and *m/z* 1035.0 resulted from a further loss of a KDO residue. Based on molecular weights and established chemical structures, the corresponding chemical composition for observed ions can be derived (Table 1). Due to the sensitivity and mild conditions of this method, minor changes in glycan structure that were not previously reported have been detected. The glycoforms corresponding to structures B and C were also detected as minor glycoforms in the large-scale sample (Figs. 3, 4), although the major glycoform populations were depicted in structure A. However, in five and ten colony samples the major glycoforms were found to be structure C. We suggest that phase-variable genes are responsible for the synthesis of different outer core [7]. Maybe some phase variants were accidentally selected when isolate colonies were picked, resulting in a different glycoform distribution compared with the major glycoforms observed for the full plate. In any case, further studies are underway to confirm and characterize the structures.

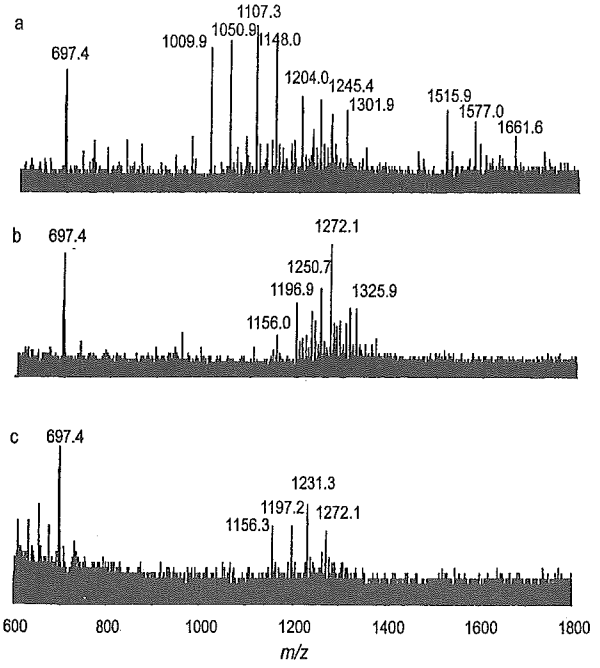


Figure 7. EA-OTLC-MS analysis of the *O*-deacylated LOS *C. jejuni* strain GC149. Extracted spectra are from 50 (a), 10 (b), and 5 colonies (c). All the spectra are the average of six scans. Other conditions were the same as in Fig. 4.

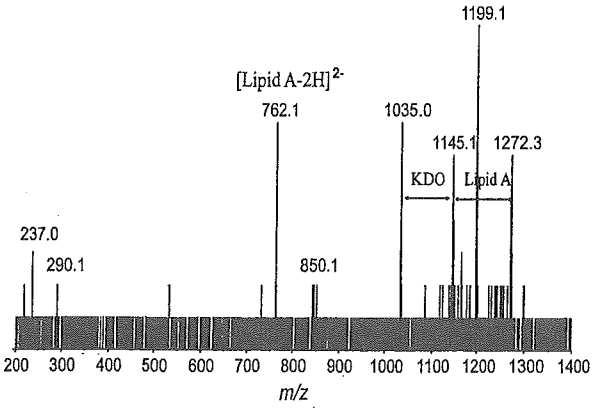


Figure 8. EA-OTLC-MS/MS analysis of the *O*-deacylated LOS from *C. jejuni* strain GC149 (ten colonies), product ion spectrum of *m/z* 1272.0 (average spectra of six scans). Other conditions were the same as in Fig. 4. E_{lab}, 120 eV (laboratory frame reference).

4 Concluding remarks

The presence of sialic acids and phosphate-containing substituents, including *P*, *P*Etn, *PP*Etn, as well as the expression of GlcN3N contribute to the structural variability of LOS molecules expressed by *C. jejuni*. The *O*-deacylation method prevents the destruction of important constituents that are acid sensitive, such as sialic acid, which tend to be lost during the mild acid hydrolysis routinely used to isolate LOS core. Changes in the glucosamine backbone and in phosphorylation of the lipid A could be easily detected. MS/MS provides a powerful tool to probe structural feature of *O*-deacylated LOS, such as sialic acids, and lipid A backbone structure. However, the lower charge state facilitates the determination of the existence of oligosialic acids. EA-OTLC-MS provides a sensitive technique to characterize *O*-deacylated LOS from as few as five colonies. The proposed method is rapid, reliable, and will assist further investigation of these important structures. This would enable researchers to understand the role of LOS in survival in multiple environments and cause of gastrointestinal disease in humans.

The authors would like to thank Dr. Eleonora Altman and Nicole Chan for review of the manuscript.

Received February 18, 2005

5 References

[1] Frost, J. A., *J. Appl. Microbiol.* 2001, *90*, 85s–95s.

[2] Prendergast, M. M., Moran, A. P., *J. Endotoxin. Res.* 2000, *6*, 341–359.

[3] Yuki, N., Susuki, K., Koga, M., Nishimoto, Y., Odaka, M., Hirata, K., Taguchi, K., Miyatake, T., Furukawa, K., Kobata, T., Yamada, M., *Proc. Natl. Acad. Sci. USA* 2004, *101*, 11404–11409.

[4] Godschalk, P. C. R., Heikema, A. P., Gilbert, M., Komagamine, T., Ang, C. W., Glerum, J., Brochu, D., Li, J., Yuki, N., Jacobs, B. C., van Belkum, A., Endtz, H. P., *J. Clin. Invest.* 2004, *114*, 1659–1665.

[5] Gilbert, M., Godschalk, P. C. R., Karwaski, M.-R., Ang, C. W., van Belkum, A., Li, J., Wakarchuk, W. W., Endtz, H. P., *Infect. Immun.* 2004, *72*, 1162–1165.

[6] Koga, M., Gilbert, M., Li, J., Koike, S., Takahashi, M., Furukawa, K., Hirata, K., Yuki, N., *Neurology* 2005, *64*, 1605–1611.

[7] Gilbert, M., Karwaski, M. F., Bernatchez, S., Young, N. M., Taboada, E., Michniewicz, J., Cunningham, A. M., Wakarchuk, W. W., *J. Biol. Chem.* 2002, *277*, 327–337.

[8] Szymanski, C. M., St. Michael, F., Jarrell, H. C., Li, J., Gilbert, M., Larocque, S., Brisson, J.-R., *J. Biol. Chem.* 2003, *278*, 24509–24520.

[9] Cox, A. D., Wright, J. C., Li, J., Hood, D. W., Moxon, E. R., Richards, J. C., *J. Bacteriol.* 2003, *185*, 3270–3277.

[10] Li, J., Cox, A. D., Hood, D. W., Moxon, E. R., Richards, J. C., *Electrophoresis* 2004, *25*, 2017–2025.

[11] Bouchet, V., Hood, D. W., Li, J., Brisson, J.-R., Randle, G. A., Martin, A., Li, Z., Goldstein, R., Schweda, E. K. H., Pelton, S. I., Richards, J. C., Moxon, E. R., *Proc. Natl. Acad. Sci. USA* 2003, *100*, 8898–8903.

[12] Gaucher, S. P., Cancilla, M. T., Phillips, N. J., Gibson, B. W., Leary, J. A., *Biochemistry* 2000, *39*, 12406–12414.

[13] Lee, N. G., Sunshine, M. G., Engstrom, J. J., Gibson, B. W., Apicella, M. A., *J. Biol. Chem.* 1995, *270*, 27151–27159.

[14] Moran, A. P., Zahringer, U., Seydel, U., Scholz, D., Stutz, P., Rietschel, E. T., *Eur. J. Biochem.* 1991, *198*, 459–469.

[15] Moran, A. P., Scholz, D., Zahringer, U., *Carbohydr. Res.* 1992, *231*, 309–316.

[16] Li, J., Purves, R. W., Richards, J. C., *Anal. Chem.* 2004, *76*, 4676–4683.

[17] Li, J., Cox, A. D., Hood, D. W., Schweda, E. K. H., Moxon, E. R., Richards, J. C., *Mol. BioSyst.* 2005, *1*, 46–52.

[18] Thibault, P., Li, J., Martin, A., Richards, J. C., Hood, D. W., Moxon, E. R., in: Burlingame, A. L., Carr, S. A., Baldwin, M. A. (Eds.), *Mass Spectrometry in Biology and Medicine*, Humana Press, Totowa, NJ 1998, pp. 439–462.

[19] Li, J., Martin, A., Richards, J. C., Wakarchuk, W. W., vander Wilp, W., Thibault, P., *J. Chromatogr. A* 1998, *817*, 325–336.

[20] Liu, Z., Zou, H., Ye, M., Ni, J., Zhang, Y., *Electrophoresis* 1999, *20*, 2891–2897.

[21] Zhu, X., Kamande, M. W., Thiam, S., Kapnissi, C. P., Mwongela, S. M., Warner, I. M., *Electrophoresis* 2004, *25*, 562–568.

[22] Hautala, J. T., Wiedmer, S. K., Riekkola, M.-L., *Electrophoresis* 2005, *26*, 176–186.

[23] Kapnissi-Christodoulou, C. P., Zhu, X., Warner, I. M., *Electrophoresis* 2004, *24*, 3917–3934.

[24] Yang, L., Guihen, E., Holmes, J. D., Loughran, M., O'Sullivan, G. P., Glennon, J. D., *Anal. Chem.* 2005, *77*, 1840–1846.

[25] Takahashi, M., Koga, M., Yokoyama, K., Yuki, N., *J. Clin. Microbiol.* 2005, *43*, 335–339.

ELSEVIER

Carbohydrate mimicry: a new paradigm of autoimmune diseases

Nobuhiro Yuki

Molecular mimicry of microbial components by self components is thought to be the mechanism that accounts for the antigen and tissue specificity of immune responses in post-infectious autoimmune diseases. Little direct evidence exists, and research in this area has focused principally on T cell mediated anti-peptide responses, rather than on humoral responses to carbohydrate structures. Guillain–Barré syndrome, the most frequent cause of acute neuromuscular paralysis, sometimes occurs after *Campylobacter jejuni* enteritis. Recent studies have revealed that carbohydrate mimicry of the bacterial lipo-oligosaccharide by the human ganglioside is an important cause of the syndrome. This new concept that carbohydrate mimicry can cause an autoimmune disease provides a clue to inducing the resolution of pathogenesis of other immune-mediated diseases.

Addresses
Department of Neurology, Dokkyo University School of Medicine, Kitakobayashi 880, Mibu, Shimotsuga, Tochigi 321-0293, Japan

Corresponding author: Yuki, Nobuhiro (yuki@dokkyomed.ac.jp)

Current Opinion in Immunology 2005, 17:577–582

This review comes from a themed issue on
Autoimmunity
Edited by Jean-François Bach and Shimon Sakaguchi

0952-7915/$ – see front matter

DOI 10.1016/j.coi.2005.09.004

Introduction

Molecular mimicry has been proposed as a pathogenic mechanism for autoimmune disease. The hypothesis is based on the epidemiological, clinical and experimental evidence of an association of infectious agents with autoimmune disease and observed cross-reactivity of immune agents with host 'self' antigens and microbial determinants. Many studies are consistent with the mimicry hypothesis, but none have convincingly demonstrated that mimicry is an important mechanism in the development of autoimmune disease in humans [1]. No replicas of human autoimmune diseases have been obtained by immunization of animals with a mimic of component of an infectious agent associated with epidemiological evidence between the disease and the agent.

Guillain–Barré syndrome (GBS), characterized by limb weakness and areflexia is a typical post-infectious autoimmune disease [2]. Since the near-elimination of poliomyelitis in the world, GBS has become the most frequent cause of acute flaccid paralysis. Most GBS patients have had gastrointestinal or upper respiratory symptoms one to two weeks before the onset of neurological symptoms. The Gram-negative bacterium *Campylobacter jejuni*, a leading cause of acute gastroenteritis in humans, is the most frequent antecedent pathogen. This review shows that carbohydrate mimicry is a cause of GBS, and that the bacterial gene polymorphism is a determinant of the clinical features. Both are new paradigms in the molecular pathogenesis of autoimmune diseases.

A true case of molecular mimicry

Four criteria must be satisfied to conclude that a disease is triggered by molecular mimicry [3°]: first, an epidemiological association between the infectious agent and the immune-mediated disease; second, the identification of T cells or antibodies directed against the patient's target antigens; third, the identification of microbial mimics of the target antigen; and forth, reproduction of the disease in an animal model. As reviewed here, GBS subsequent to *C. jejuni* enteritis fulfils all four criteria and provides the first verification that molecular mimicry is a cause of human autoimmune diseases.

Epidemiological association of *Campylobacter jejuni* with Guillain–Barré syndrome

A case-control study detected evidence of recent *C. jejuni* infection in 26% of patients with GBS in comparison to 2% of the household controls (a member of the patient's household) and 1% of the age-matched hospital controls [4]. This study established an epidemiological association between *C. jejuni* infection and GBS. *C. jejuni*-isolated GBS was most common in 10–30 year-old individuals, and the male:female ratio was 1.7:1 [5]. The median latent period between antecedent symptoms and the onset of neuropathy was 10 days.

GBS was originally considered to be a demyelinating disease of peripheral nerves. This view of GBS was shaped largely by what is known of its laboratory analogue, the experimental allergic neuritis induced by immunization with P2 protein (a component of peripheral nerve myelin). Now the presence of a primary axonal GBS, acute motor axonal neuropathy (AMAN), is widely recognized through findings of autopsy studies [6,7]. *C. jejuni* infection is associated with AMAN, but not with demyelinating GBS [8].

Autoantibodies against gangliosides in Guillain–Barré syndrome

Autoantibodies against the surface components of peripheral nerves were originally considered to be pathogenic

substances that induce GBS because plasma exchange facilitates the rate of recovery [2]. Gangliosides constitute a large family predominantly made up of cell-surface glycosphingolipids bearing a ceramide moiety anchored in the external leaflet of the lipid bilayer and a sialylated oligosaccharide core exposed extracellularly.

In AMAN, IgG is deposited on the axolemma of the spinal anterior roots [7]. This indicates that IgG, which binds effectively with complement components, is an important factor in the development of AMAN. Patients who developed AMAN subsequent to *C. jejuni* enteritis had IgG antibody against GM1, and their autoantibody titers decreased with the clinical course [9]. By contrast, patients who had had *C. jejuni* enteritis but no neurological disorder did not have the autoantibodies. GD1a as well as GM1 is an autoantigen for IgG antibodies in patients with AMAN subsequent to *C. jejuni* enteritis [10].

Campylobacter jejuni mimic of gangliosides

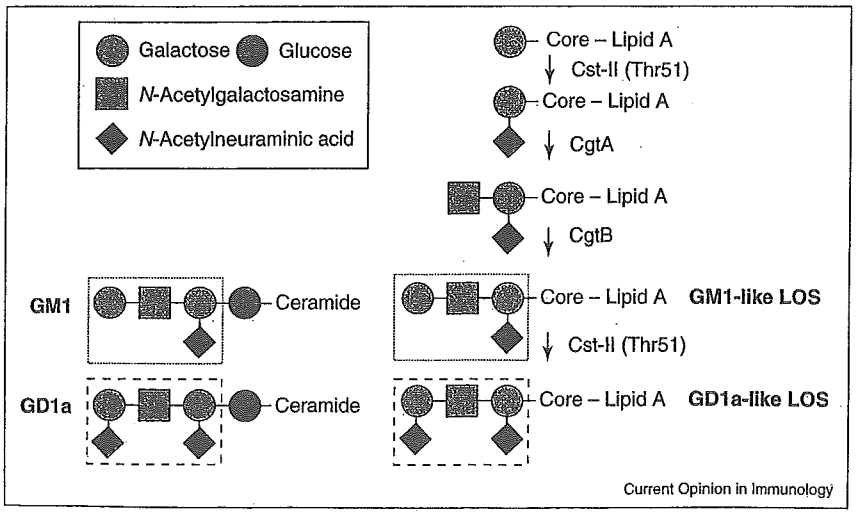
Lipo-oligosaccharide (LOS) is one of the most important cell-surface structures expressed by *C. jejuni*. One *C. jejuni* strain (CF90-26), isolated from an AMAN patient carrying anti-GM1 IgG antibodies, expresses an oligosaccharide structure (Gal β1–3 GalNAc β1–4 [NeuAc α2–3] Galβ), which protrudes from the LOS core (Figure 1) [11]. This terminal structure is identical to the terminal tetrasaccharide of the GM1 ganglioside. This was the first definitive evidence of molecular mimicry between human nerve tissue and *C. jejuni*. This strain also carries a GD1a-like LOS (Figure 1) [12]. Another *C. jejuni* strain (16971.94GSH), isolated from a patient with GBS, carried a GM1-like LOS [13]. One *C. jejuni* strain (ATCC 43446) from an enteritis patient also had GM1-like and GD1a-like LOSs [14].

Animal models of Guillain–Barré syndrome

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 or an isolated GM1, some patients developed AMAN, and anti-GM1 IgG antibody was detected in these patients [15]. An AMAN model was established by sensitization of Japanese white rabbits with a bovine brain ganglioside mixture or an isolated GM1 [16]. 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 paralyzed rabbits showed macrophage infiltration in the periaxonal space [17]. Surrounding myelin sheaths were almost intact. These findings correspond well with pathological findings for human AMAN [6,7]. This AMAN rabbit model was also reproducible in New Zealand white rabbits [18].

The most straightforward way to verify whether molecular mimicry between microbes and autoantigens causes GBS is to establish a GBS model by immunizing animals with components of antecedent infectious agents. An

Figure 1



Carbohydrate mimicry of GM1/GD1a gangliosides, *Campylobacter jejuni* lipo-oligosaccharides (LOSs) and enzymatic synthesis of the GM1-like and GD1a-like LOSs. The structure of the terminal tetrasaccharides of GM1-like LOS is identical to that of GM1 (shown by the dotted lines). The structure of the terminal pentasaccharides of GD1a-like LOS is identical to that of GD1a (shown by the dashed lines). GM1-like and GD1a-like LOSs are synthesized by sialyltransferase Cst-II (Thr51), *N*-acetylgalactosaminyl-transferase (CgtA) and galactosyltransferase (CgtB).

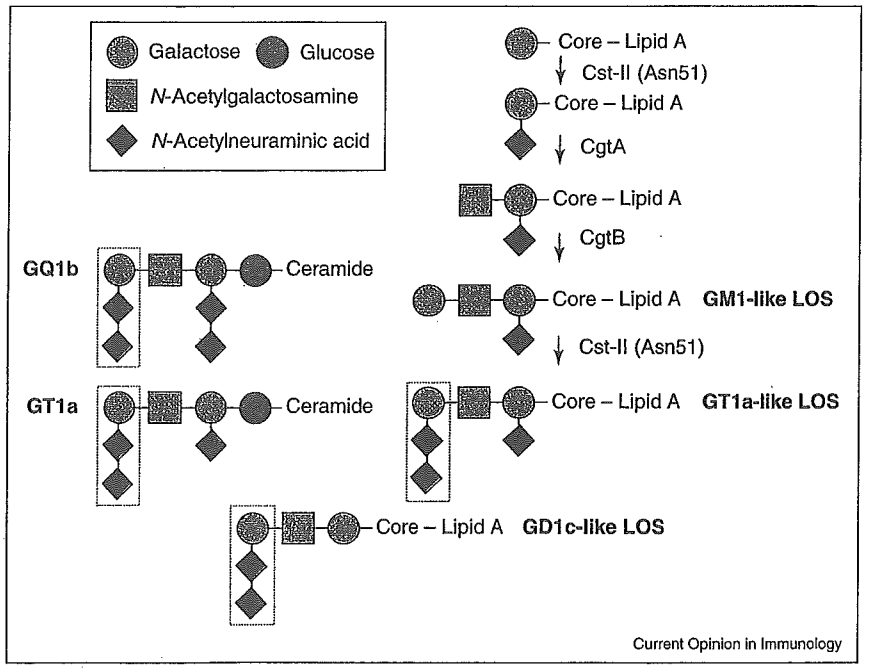
AMAN model was established by the immunization of Japanese white rabbits with *C. jejuni* LOS bearing a GM1-like structure [19••]. Following sensitization with this GM1-like LOS, rabbits developed high anti-GM1 IgG antibody titres and subsequent flaccid limb weakness. Their nerve roots had occasional 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, which are compatible with the features of human AMAN, are evidence that rabbits inoculated with *C. jejuni* LOS 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.

Campylobacter jejuni genes associated with Guillain–Barré syndrome

As stated, ganglioside mimicry of *C. jejuni* LOS is a cause of GBS. Ganglioside-like LOS is synthesized by sialyltransferase Cst-II, *N*-acetylgalactosaminyl-transferase CgtA, and galactosyltransferase CgtB (Figures 1 and 2) [20]. Compared with gastroenteritis-related isolates, GBS-related *C. jejuni* isolates have a strong association with the expression of GD1a mimicry [21]. Some genes (*cst-II*, *cgtA* and *cgtB*) that are involved in ganglioside mimicry are also associated with GBS-related bacterial strains. These LOS biosynthesis genes cluster at the LOS biosynthesis gene locus [22]. A specific type of gene locus (called a class A gene locus), which includes these three genes, is associated both with GBS and with the GM1-like LOS [23••]. We have shown that isolates from GBS patients have specific LOS biosynthesis gene loci, which contain *cst-II* or *cst-III* more frequently than loci from enteritis patients (102/106 [96%] versus 72/103 [70%]; the difference is statistically significant), and that the GBS isolates expressed the GM1 or GD1a epitope, or both (M Koga and N Yuki, unpublished).

The *cst-II* gene encodes an enzyme that transfers sialic acid to the LOS, and *neuA1* encodes an enzyme that synthesizes the donor (CMP-sialic acid) used by the Cst-II sialyltransferase [20]. Because both genes are involved in LOS sialylation, they are essential for ganglioside-like LOS synthesis. Mutants of *C. jejuni* that lack these genes have been made and analysed [23••]. Whereas a mixture of GM1-like and GD1a-like structures were identified in wild-type *C. jejuni* strains isolated from GBS patients, neither structure was present in the mutants. Both the *cst-II* and *neuA1* 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-naïve hosts that

Figure 2



Carbohydrate mimicry of GQ1b/GT1a gangliosides, *Campylobacter jejuni* lipo-oligosaccharides (LOSs) and enzymatic synthesis of the GT1a-like LOSs. The structure of the terminal trisaccharides of GQ1b is identical to that of GT1a, GT1a-like LOS and GD1c-like LOS (shown by the dotted line). GT1a-like LOS is synthesized by sialyltransferase Cst-II (Asn51), *N*-acetylgalactosaminyl-transferase (CgtA) and galactosyltransferase (CgtB).

can be used to obtain high-titre anti-ganglioside antibody responses. Immunization with the wild-type strain induced an anti-GD1a IgG antibody response in these mice, whereas immunization with the mutant strains did not. This shows that the genes involved in LOS sialylation are essential for the induction of anti-ganglioside antibodies.

A bacterial gene (*cst-II*) polymorphism as a determinant of neurological features

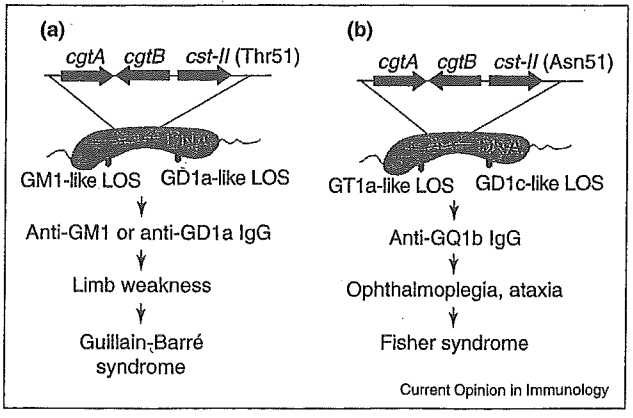
The reason why a certain microbial infection can induce the development of different autoimmune diseases has yet to be clarified. For example, group A streptococcal infections can induce the development of acute rheumatic fever in some patients and acute glomerulonephritis in others; however, the molecular pathogenesis is unknown [24]. The mechanism by which *C. jejuni* infection induces the development of GBS in some patients and Fisher syndrome (FS) in others has recently been clarified [25••].

Whereas GBS is characterized by limb weakness and areflexia, FS is characterized by ophthalmoplegia, ataxia and areflexia [2]. FS is regarded as a clinical variant of GBS because they sometimes overlap (some FS patients develop GBS during the illness). Although the presence of anti-GM1 and anti-GD1a IgG antibodies is closely related to AMAN, the presence of IgG antibodies to GQ1b, which is expressed in the oculomotor nerves and primary sensory neurons, are highly specific to patients with FS [26,27]. The anti-GQ1b IgG antibody cross-reacts with GT1a, indicating that the autoantibody recognizes the terminal trisaccharides common to GQ1b and GT1a (Figure 2). A case-control study showed serologic evidence of *C. jejuni* infections in FS patients was present significantly more often than in the sex- and age-matched hospital controls (21% versus 3%; [28•]). One *C. jejuni* strain (CF93-6) carried a GT1a-like LOS that had been isolated from a patient with FS. Another *C. jejuni* strain (PG836) isolated from an FS patient carried a GD1c-like LOS [29]. Both GT1a-like and GD1c-like LOS have a trisaccharide residue that is also common to GQ1b ganglioside (Figure 2).

Variation in the nucleotide sequence of *cst-II* might affect enzymatic activity; Cst-II (Thr51) has only α-2,3-sialyltransferase activity (monofunctional) and can make GM1-like and GD1a-like LOSs, whereas Cst-II (Asn51) has both α-2,3- and α-2,8-sialyltransferase activities (bifunctional) and can make GT1a-like and GD1c-like LOSs (Figure 2) [22]. Neuropathic strains more frequently have *cst-II* than enteritic strains [25••]. Strains with *cst-II* (Thr51) have the GM1 and GD1a epitopes, whereas those with *cst-II* (Asn51) regularly express the GQ1b epitope. Patients infected with *C. jejuni* (Thr51) more frequently than *C. jejuni* (Asn51) were positive for anti-GM1 and anti-GD1a IgG and had limb weakness, and GBS was diagnosed. Patients infected with *C. jejuni* (Asn51) were more often positive for anti-GQ1b IgG and had ophthalmoplegia and ataxia, and FS and the related conditions were diagnosed.

These results have presented a new paradigm that bacterial genetic polymorphism determines the clinical presentation of autoimmune diseases (Figure 3).

Figure 3



Campylobacter jejuni gene polymorphism as a determinant of clinical neuropathies after infection by the bacterium. **(a)** *C. jejuni* carrying *cst-II* (Thr51) can express GM1-like or GD1a-like LOS on its cell surfaces. Infection by such *C. jejuni* strains can induce anti-GM1 or anti-GD1a IgG production in certain patients. Anti-GM1 or anti-GD1a IgG antibodies bind to GM1 or GD1a, respectively; these are expressed on motor nerves in the four limbs. This binding induces the development of Guillain–Barré syndrome. **(b)** By contrast, *C. jejuni* carrying *cst-II* (Asn51) can express GT1a-like or GD1c-like LOS on its cell surfaces. Infection by such *C. jejuni* strains induces anti-GQ1b IgG production in certain patients. Anti-GQ1b IgG antibody binds to GQ1b, which is expressed on the oculomotor nerves and primary sensory neurons. This induces the development of Fisher syndrome.

Conclusions

Convincing evidence has shown that carbohydrate mimicry is a cause of GBS/FS subsequent to *C. jejuni* enteritis. Moreover, the bacterial gene responsible for this has been determined (*cst-II*). Only a minority of those who have *C. jejuni* enteritis develop GBS [30], and no host susceptibility genes associated with the development of GBS have yet been identified. The lipopolysaccharide receptors CD14 and Toll-like receptor 4 (TLR4) are important in antigen presentation and intracellular signalling, but the functional polymorphisms in *CD14* and *TLR4* are not associated with susceptibility to *C. jejuni*-associated GBS [31]. A genome-wide search of single nucleotide polymorphisms could identify the host susceptibility genes.

Acknowledgements

I thank M Gilbert for critical reading of the manuscript. The work that contributed to this review is supported in part by a grant-in-aid for Scientific Research (B) (KAKENHI 14370210) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; a Health Science Research Grant (Research on Brain Science) from the Ministry of Health and Welfare of Japan; and, a grant from the Human Frontier Science Program (RGP0038/2003-C).

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Marrack P, Kappler J, Kotzin BL: **Autoimmune disease: why and where it occurs.** *Nat Med* 2001, **7**:899-905.

2. Yuki N: **Infectious origins of, and molecular mimicry in, Guillain–Barré and Fisher syndromes.** *Lancet Infect Dis* 2001, **1**:29-37.

3. • Ang CW, Jacobs BC, Laman JD: **The Guillain–Barré syndrome: a true case of molecular mimicry.** *Trends Immunol* 2004, **25**:61-66.
This review shows that GBS is an excellent model for the clarification of both the host and microbial aspects of molecular mimicry.

4. Rees JH, Soudain SE, Gregson NA, Hughes RAC: ***Campylobacter jejuni* infection and Guillain–Barré syndrome.** *N Engl J Med* 1995, **333**:1374-1379.

5. Takahashi M, Koga M, Yokoyama K, Yuki N: **Epidemiology of *Campylobacter jejuni* isolated from patients with Guillain–Barré and Fisher syndromes in Japan.** *J Clin Microbiol* 2005, **43**:335-339.

6. McKhann GM, Cornblath DR, Griffin JW, Ho TW, Li CY, Jiang Z, Wu HS, Zhaori G, Liu Y, Jou LP *et al.*: **Acute motor axonal neuropathy: a frequent cause of acute flaccid paralysis in China.** *Ann Neurol* 1993, **33**:333-342.

7. Hafer-Macko C, Hsieh ST, Li CY, Ho TW, Sheikh K, Cornblath DR, McKhann GM, Asbury AK, Griffin JW: **Acute motor axonal neuropathy: an antibody-mediated attack on axolemma.** *Ann Neurol* 1996, **40**:635-644.

8. Kuwabara S, Ogawara K, Misawa S, Koga M, Mori M, Hiraga A, Kanesaka T, Hattori T, Yuki N: **Does *Campylobacter jejuni* infection elicit "demyelinating" Guillain–Barré syndrome?** *Neurology* 2004, **63**:529-533.

9. Yuki N, Yoshino H, Sato S, Miyatake T: **Acute axonal polyneuropathy associated with anti-GM1 antibodies following *Campylobacter* enteritis.** *Neurology* 1990, **40**:1900-1902.

10. Ho TW, Willison HJ, Nachamkin I, Li CY, Veitch J, Ung H, Wang GR, Liu RC, Cornblath DR, Asbury AK *et al.*: **Anti-GD1a antibody is associated with axonal but not demyelinating forms of Guillain–Barré syndrome.** *Ann Neurol* 1999, **45**:168-173.

11. Yuki N, Taki T, Inagaki F, Kasama T, Takahashi M, Saito K, Handa S, Miyatake T: **A bacterium lipopolysaccharide that elicits Guillain–Barré syndrome has a GM1 ganglioside-like structure.** *J Exp Med* 1993, **178**:1771-1775.

12. Yuki N, Taki T, Takahashi M, Saito K, Tai T, Miyatake T, Handa S: **Penner's serotype 4 of *Campylobacter jejuni* has a lipopolysaccharide that bears a GM1 ganglioside epitope as well as one that bears a GD1a epitope.** *Infect Immun* 1994, **62**:2101-2103.

13. Prendergast MM, Lastovica AJ, Moran AP: **Lipopolysaccharides from *Campylobacter jejuni* O:41 strains associated with Guillain–Barré syndrome exhibit mimicry of GM1 ganglioside.** *Infect Immun* 1998, **66**:3649-3655.

14. Aspinall GO, McDonald AG, Pang H, Kurjanczyk LA, Penner JL: **Lipopolysaccharides of *Campylobacter jejuni* serotype O:19: structures of core oligosaccharide regions from the serostrain and two bacterial isolates from patients with the Guillain–Barré syndrome.** *Biochemistry* 1994, **33**:241-249.

15. Ilia I, Ortiz N, Gallard E, Juarez C, Grau JM, Dalakas MC: **Acute axonal Guillain–Barré syndrome with IgG antibodies against motor axons following parenteral gangliosides.** *Ann Neurol* 1995, **38**:218-224.

16. Yuki N, Yamada M, Koga M, Odaka M, Susuki K, Tagawa Y, Ueda S, Kasama T, Ohnishi A, Hayashi S *et al.*: **Animal model of axonal Guillain–Barré syndrome induced by sensitization with GM1 ganglioside.** *Ann Neurol* 2001, **49**:712-720.

17. Susuki K, Nishimoto Y, Yamada M, Baba M, Ueda S, Hirata K, Yuki N: **Acute motor axonal neuropathy rabbit model: immune attack on nerve root axons.** *Ann Neurol* 2003, **54**:383-388.

18. Susuki K, Nishimoto Y, Koga M, Nagashima T, Mori I, Hirata K, Yuki N: **Various immunization protocols for an acute motor axonal neuropathy rabbit model compared.** *Neurosci Lett* 2004, **368**:63-67.

19. •• Yuki N, Susuki K, Koga M, Nishimoto Y, Odaka M, Hirata K, Taguchi K, Miyatake T, Furukawa K, Kobata T *et al.*: **Carbohydrate mimicry between human ganglioside GM1 and *Campylobacter jejuni* lipooligosaccharide causes Guillain–Barré syndrome.** *Proc Natl Acad Sci USA* 2004, **101**:11404-11409.
This study provides convincing evidence that molecular mimicry is a cause of GBS subsequent to *C. jejuni* enteritis. Moreover, it is the first to verify that molecular mimicry is a cause of human autoimmune disease.

20. Gilbert M, Brisson JR, Karwaski MF, Michniewicz J, Cunningham AM, Wu Y, Young NM, Wakarchuk WW: **Biosynthesis of ganglioside mimics in *Campylobacter jejuni* OH4384: identification of the glycosyltransferase genes, enzymatic synthesis of model compounds, and characterization of nanomole amounts by 600-MHz ^{1}H and ^{13}C NMR analysis.** *J Biol Chem* 2000, **275**:3896-3906.

21. Nachamkin I, Liu J, Li M, Ung H, Moran AP, Prendergast MM, Sheikh K: ***Campylobacter jejuni* from patients with Guillain–Barré syndrome preferentially expresses a GD1a-like epitope.** *Infect Immun* 2002, **70**:5299-5303.

22. Gilbert M, Karwaski MF, Bernatchez S, Young NM, Taboada E, Michniewicz J, Cunningham AM, Wakarchuk WW: **The genetic bases for the variation in the lipo-oligosaccharide of the mucosal pathogen, *Campylobacter jejuni*: biosynthesis of sialylated ganglioside mimics in the core oligosaccharide.** *J Biol Chem* 2002, **277**:327-337.

23. •• Godschalk PC, Heikema AP, Gilbert M, Komagamine T, Ang CW, Glerum J, Brochu D, Li J, Yuki N, Jacobs BC *et al.*: **The crucial role of *Campylobacter jejuni* genes in anti-ganglioside antibody induction in Guillain–Barré syndrome.** *J Clin Invest* 2004, **114**:1659-1665.
The authors demonstrate for the first time that specific bacterial genes are required for the induction of anti-ganglioside antibodies.

24. Stollerman GH: **Rheumatic fever.** *Lancet* 1997, **349**:935-942.

25. •• Koga M, Takahashi M, Masuda M, Hirata K, Yuki N: ***Campylobacter* gene polymorphism as a determinant of**

clinical features of Guillain–Barré syndrome. *Neurology*, in press.
This paper presents a new paradigm that bacterial genetic polymorphisms determine the clinical presentation of autoimmune diseases.

26. Chiba A, Kusunoki S, Obata H, Machinami R, Kanazawa I: **Serum anti-GQ1b IgG antibody is associated with ophthalmoplegia in Miller Fisher syndrome and Guillain–Barré syndrome: clinical and immunohistochemical studies.** *Neurology* 1993, **43**:1911-1917.

27. Kusunoki S, Chiba A, Kanazawa I: **Anti-GQ1b IgG antibody is associated with ataxia as well as ophthalmoplegia.** *Muscle Nerve* 1999, **22**:1071-1074.

28. • Koga M, Gilbert M, Li J, Koike S, Takahashi M, Furukawa K, Hirata K, Yuki N: **Antecedent infections in Fisher syndrome: a common pathogenesis of molecular mimicry.** *Neurology* 2005, **64**:1605-1611.
This study shows that not only *C. jejuni* but also *Haemophilus influenzae* are related to FS development, and that production of anti-GQ1b autoantibody is mediated by the GQ1b-mimicking LOS on those bacteria.

29. Shin JE, Ackloo S, Mainkar AS, Monteiro MA, Pang H, Penner JL, Aspinall GO: **Lipo-oligosaccharides of *Campylobacter jejuni* serotype O:10: structures of core oligosaccharide regions from a bacterial isolate from a patient with the Miller Fisher syndrome and from the serotype reference strain.** *Carbohydr Res* 1997, **305**:223-232.

30. McCarthy N, Giesecke J: **Incidence of Guillain–Barré syndrome following infection with *Campylobacter jejuni*.** *Am J Epidemiol* 2001, **153**:610-614.

31. Geleijns K, Jacobs BC, Van Rijs W, Tio-Gillen AP, Laman JD, van Doorn PA: **Functional polymorphisms in LPS receptors *CD14* and *TLR4* are not associated with disease susceptibility or *Campylobacter jejuni* infection in Guillain–Barré patients.** *J Neuroimmunol* 2004, **150**:132-138.

Neurogenetics (2005) 6: 225–228
DOI 10.1007/s10048-005-0022-0

ORIGINAL ARTICLE

Janardan P. Pandey · Michiaki Koga · Nobuhiro Yuki

Immunoglobulin KM allotypes are associated with the prevalence of autoantibodies to GD1a ganglioside, but not with susceptibility to the disease, in Japanese patients with Guillain–Barré syndrome

Received: 28 June 2005 / Accepted: 19 September 2005 / Published online: 29 October 2005

Abstract Guillain–Barré syndrome (GBS), an autoimmune disease of the peripheral nervous system, is associated with antecedent *Campylobacter jejuni* infection. GM and KM allotypes—genetic markers of immunoglobulin γ and κ chains, respectively—are implicated in the etiopathogenesis of several autoimmune diseases. To determine if GM/KM phenotypes are associated with GBS and influence antibody responses to *C. jejuni* and to GM1 and GD1a gangliosides, 72 Japanese GBS patients and 73 controls were allotyped for several GM and KM markers. Sera from patients were characterized for antibodies to *C. jejuni*, GM1, and GD1a. The distribution of KM phenotypes was significantly different in patients with anti-GD1a ganglioside antibodies from those who lacked these antibodies (P=0.029). No other significant associations were found. These results suggest that KM allotypes are not risk factors for developing GBS, but contribute significantly to the generation of autoimmune responses to GD1a ganglioside in patients with this disease.

Keywords Guillain–Barré syndrome · GM/KM allotypes · Gangliosides · Linkage disequilibrium · Autoantibodies

Introduction

Guillain–Barré syndrome (GBS), an inflammatory autoimmune disease of the peripheral nervous system, is strongly associated with antecedent *Campylobacter jejuni* infection [1]. However, only a minority of the infected individuals develops the disease [2], implying a role for the host genetic factors in conferring susceptibility to this syndrome. Results of our investigation in a Norwegian population have shown that particular KM allotypes—the hereditary antigenic determinants of immunoglobulin (Ig) κ chains—are significantly associated with susceptibility/resistance to this syndrome [3]. KM allotypes could play a role in susceptibility to GBS by influencing the ganglioside mimicry of *C. jejuni* lipo-oligosaccharides, which contributes to the etiology of the disease [4, 5]. Since KM gene frequency differs significantly among racial groups [6], it is important to determine whether the associations observed in Norwegian Caucasians are present in other racial groups as well. In the present investigation, our aim was to determine whether KM and GM (genetic markers of γ chains) phenotypes were associated with GBS in a Japanese population. We also determined whether these markers were associated with the prevalence of autoantibodies to GM1 and GD1a gangliosides. In addition, we investigated the possible influence of GM and KM allotypes on the prevalence of IgG and IgA antibodies to *C. jejuni*.

J. P. Pandey (✉)
Department of Microbiology and Immunology,
Medical University of South Carolina,
Charleston, SC 29425-2230, USA
e-mail: pandeyj@musc.edu
Tel.: +1-843-7924360
Fax: +1-843-7924882

M. Koga · N. Yuki
Department of Neurology,
Dokkyo University School of Medicine,
Tochigi, Japan

Subjects and methods

Study population

The study population consisted of 72 Japanese GBS patients and 73 controls (disease controls without autoimmune disease and healthy persons). All patients fulfilled the published criteria for GBS [7]. This study was approved by the Institutional Review Board for human research of the Medical University of South Carolina.

GM and KM allotyping

Serum samples from patients and controls were typed for G1M (1/a, 2/x, 3/f, 17/z), G2M (23/n), G3M (5/b1, 6/c3, 13/b3, 21/g), and KM1 and 3 allotypes by a standard hemag-

Table 1 Distribution of GM and KM phenotypes in GBS patients and controls

Phenotypes	GBS patients *n* (%)	Controls *n* (%)
GM1, 17 21	34 (47.2)	25 (34.2)
GM1, 2, 17 21	18 (25.0)	27 (37.0)
Other GM	20 (27.8)	21 (28.8)
KM1	9 (12.5)	6 (8.2)
KM3	38 (52.8)	47 (64.4)
KM1, 3	25 (34.7)	20 (27.4)

glutination-inhibition method [8, 9]. The notation follows the international system for human gene nomenclature [10], in which haplotypes and phenotypes are written by grouping together the markers that belong to each IgG subclass, by the numerical order of the marker and of the subclass; markers belonging to different subclasses are separated by a space, while allotypes within a subclass are separated by commas.

Antibody determinations

IgG antibodies to *C. jejuni*, GM1, and GD1a were measured by enzyme-linked immunosorbent assays described elsewhere [11].

Statistical analyses

The distribution of GM and KM phenotype frequencies was analyzed using Pearson's χ^2 test, except when cell counts were less than or equal to 5; in the latter case, data were analyzed by Fisher's two-tailed exact test. Statistical significance was defined as $P<0.05$. GM phenotypes whose prevalence in the sample was less than 5% were pooled (other GM), so as not to have a test with too many degrees of freedom.

Results

The distribution of GM and KM phenotypes in GBS patients and controls is given in Table 1. None of the GM or KM phenotypes was associated with GBS. The distribution of GM and KM phenotypes in patients with and without antibodies to *C. jejuni* and to GM1 and GD1a gangliosides is presented in Table 2. The distribution of KM phenotypes was significantly different in patients positive for anti-GD1a ganglioside antibodies from those who lacked these antibodies. Of patients with the antibodies to GD1a, 76% were KM 3 homozygotes, whereas only 45% of the subjects lacking these antibodies possessed this phenotype (P=0.029). All KM1 homozygotes lacked antibodies to the GD1a ganglioside. No other significant associations were found.

Discussion

Results presented here show a significant association between homozygosity for the KM3 allele and the presence of anti-GD1a ganglioside antibodies. Molecular mimicry between the lipo-oligosaccharides of *C. jejuni* and gangliosides present in peripheral nerves is the most favored mechanism underlying *C. jejuni*–GBS association [5]. The KM locus could affect autoimmune responsiveness to the GD1a ganglioside indirectly through its regulatory influence on the generation of cross-reacting antibodies to *C. jejuni*. Although we did not find an association between KM phenotypes and antibody responses to the outer membrane protein of *C. jejuni*, it is possible that such a relationship exists between these determinants and antibody responses to the lipo-oligosaccharide of *C. jejuni* that is structurally similar to the GD1a ganglioside. Studies to determine the role of KM allotypes in immunity to such cross-reacting lipo-oligosaccharides are warranted. Since KM allotypes have been associated with IgA antibody responses to *C. jejuni* in a US Caucasian population convalescing from acute *C. jejuni* infection [12], we sought to determine whether a relationship exists between these markers and the prevalence of IgA antibodies to *C. jejuni* in GBS patients in the present investigation. No significant associations were found (data not shown). Perhaps persons genetically predisposed to GBS recognize different *C. jejuni* epitopes from those that are immunogenic in the majority of the infected people who do not develop the disease. It appears that humoral immunity to the latter [12], but not to the former, is KM allotype associated. It might be

Table 2 Distribution of GM and KM phenotypes in GBS patients with or without IgG antibodies to *C. jejuni* and to GM1 and GD1a gangliosides

Phenotypes	*C. jejuni*		GM1		GD1a	
	Positive *n* (%)	Negative *n* (%)	Positive *n* (%)	Negative *n* (%)	Positive *n* (%)	Negative *n* (%)
GM1, 17 21	9 (40.9)	25 (50.0)	12 (50.0)	22 (45.8)	8 (47.1)	26 (47.3)
GM1, 2, 17 21	6 (27.3)	12 (24.0)	5 (20.8)	13 (27.1)	4 (23.5)	14 (25.5)
Other GM	7 (31.8)	13 (26.0)	7 (29.2)	13 (27.1)	5 (29.4)	15 (27.3)
KM1	4 (18.1)	5 (10.0)	2 (8.3)	7 (14.6)	0 (0.0)	9 (16.4)
KM3	12 (54.5)	26 (52.0)	13 (54.2)	25 (52.1)	13 (76.5)	25 (45.5)[a]
KM1, 3	6 (27.3)	19 (38.0)	9 (37.5)	16 (33.3)	4 (23.5)	21 (38.2)

[a]Fisher's exact test (two-tail), P=0.029 (KM3 vs KM1 and KM1, 3)

relevant to note that KM allotypes—in addition to their involvement in immunity to various infectious pathogens [13]—have been associated with the prevalence of several autoantibodies in other autoimmune diseases [14–17].

Although KM markers are constant (C)-region determinants, they can influence immune responsiveness associated with the variable (V) region in several ways, including direct contribution to the formation of idiotypic determinants, modulation of antibody binding affinity, linkage disequilibrium with alleles coding for the V-region epitopes. Examples of these and other possible mechanisms underlying the influence of the C region on antibody specificity are known [18–20]. A recent study has established the validity of these observations, which challenge the current central tenet of immunology that the V region is the sole determinant of antibody specificity [21]. These authors have conclusively shown that changes in the C region cause changes in the specificity of V-region identical binding sites. Amino acid substitutions associated with the allelic variation at the KM locus cause structural changes in the C region, and it follows that they could affect the V-region protein conformation, resulting in changes in antibody specificity.

Significant linkage disequilibrium between particular KM alleles located on chromosome 2, and the alleles of another as-yet-unidentified immune response gene for the GD1a ganglioside could also give rise to the associations observed here. In contrast to our findings in the Caucasian GBS patients from Norway [3], we did not find an association between KM phenotypes and GBS in the Japanese patients in the present study. The reasons for this ethnic difference in KM–GBS association are not clear. Involvement of a gene in susceptibility to a disease in one ethnic group, but not in another, may be a reflection of genetic heterogeneity, a well-documented phenomenon in polygenic diseases [22]. The KM allele frequencies in the Japanese are significantly different from that in Caucasians [6], which could potentially contribute to the differences in disease associations. Although we did not find any associations between the KM locus and GBS in the Japanese, it is possible that these determinants do contribute to the disease in this ethnic group as well, but that their role in this population is minor and detectable only in the presence of another major disease susceptibility gene—perhaps an allele of the HLA system [23]. Such interactions between KM and HLA alleles have been shown in systemic sclerosis [16], a systemic connective tissue autoimmune disease. Studies to simultaneously examine the role of KM and HLA loci in the etiopathogenesis of GBS are warranted.

To our knowledge, this is the first report of an association between a KM phenotype and autoimmune responses to gangliosides. Although this association can, at least partially, be explained by the known immunological properties of KM genes, they must be followed by confirmation in an independent study population to be of wider significance.

Acknowledgements This work was supported in part by the US Department of Energy cooperative agreement DE-FC09-02CH11109 and by a grant-in-aid for Scientific Research (B) (KAKENHI 14370210 to N.Y.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We thank Keith Rocca and Brenda Steadham for excellent technical assistance.

References

1. Yuki N (2001) Infectious origins of, and molecular mimicry in, Guillain-Barré and Fisher syndromes. Lancet Infect Dis 1:29–37
2. McCarthy N, Giesecke J (2001) Incidence of Guillain–Barré syndrome following infection with *Campylobacter jejuni*. Am J Epidemiol 153:610–614
3. Pandey JP, Vedeler CA (2003) Immunoglobulin KM genes in Guillain–Barré syndrome. Neurogenetics 4:147–149
4. Yuki N, Taki T, Takahashi M, Saito K, Tai T, Miyatake T, Handa S (1994) Penner's serotype 4 of *Campylobacter jejuni* has a lipopolysaccharide that bears a GM1 ganglioside epitope as well as one that bears a GD1a epitope. Infect Immun 62:2101–2103
5. Yuki N, Susuki K, Koga M, Nishimoto Y, Odaka M, Hirata K, Taguchi K, Miyatake T, Furukawa K, Kobata T, Yamada M (2004) Carbohydrate mimicry between human ganglioside GM1 and *Campylobacter jejuni* lipooligosaccharide causes Guillain–Barré syndrome. Proc Natl Acad Sci U S A 101:11404–11409
6. Steinberg AG, Cook CE (1981) The distribution of human immunoglobulin allotypes. Oxford University Press, New York
7. Asbury AK, Cornblath DR (1990) Assessment of current diagnostic criteria for Guillain–Barré syndrome. Ann Neurol 27:S21–S24
8. Vyas GN, Fudenberg HH, Pretty HM, Gold ER (1968) A new rapid method for genetic typing of human immunoglobulins. J Immunol 100:274–279
9. Schanfield MS, van Loghem E (1986) Human immunoglobulin allotypes. In: Weir DM (ed) Handbook of experimental immunology, 4th edn, vol 3, Genetics and molecular immunology. Blackwell, Boston, pp 94.1–94.18
10. Shows TB, McAlpine PJ, Boucheix C, Collins FS, Conneally PM, Frezal J, Gershowitz H, Goodfellow PN, Hall JG et al (1987) Guidelines for human gene nomenclature. An international system for human gene nomenclature (ISGN). Cytogenet Cell Genet 46:11–28
11. Koga M, Gilbert M, Li J, Koike S, Takahashi M, Furukawa K, Hirata K, Yuki N (2005) Antecedent infections in Fisher syndrome: a common pathogenesis of molecular mimicry. Neurology 64:1605–1611
12. Pandey JP, Blaser MJ (1986) Heterozygosity at the Km locus associated with humoral immunity to *Campylobacter jejuni*. Exp Clin Immunogenet 3:49–53
13. Pandey JP (2001) Immunoglobulin GM and KM allotypes and vaccine immunity. Vaccine 19:613–617
14. Whittingham S, Propert DN, Mackay IR (1984) A strong association between the nuclear antibody anti-La (SS-B) and the kappa chain allotype Km(1). Immunogenetics 19:295–299
15. Hoffman RW, Sharp GC, Irvin WS, Anderson SK, Hewett JE, Pandey JP (1991) Association of immunoglobulin Km and Gm allotypes with specific antinuclear antibodies and disease susceptibility among connective tissue disease patients. Arthritis Rheum 34:453–458
16. Kameda H, Pandey JP, Kaburaki J, Inoko H, Kuwana M (1998) Immunoglobulin allotype gene polymorphisms in systemic sclerosis: interactive effect of MHC class II and KM genes on anticentromere antibody production. Ann Rheum Dis 57:366–370

17. Pandey JP, Page GP, Silver RM, LeRoy EC, Bona CA (2001) Anti-fibrillin-1 autoantibodies in systemic sclerosis are GM and KM allotype restricted. Exp Clin Immunogenet 18:123–129
18. Morahan G, Berek C, Miller JFAP (1983) An idiotypic determinant formed by both immunoglobulin constant and variable regions. Nature 301:720–722
19. Moxley G, Gibbs RS (1992) Polymerase chain reaction-based genotyping for allotypic markers of immunoglobulin kappa shows allelic association of Km with kappa variable segment. Genomics 13:104–108
20. Pritsch O, Hudry-Clergeon G, Buckle M, Petillot Y, Bouvet JP, Gagnon J, Dighiero G (1996) Can immunoglobulin CH1 constant region domain modulate antigen binding affinity of antibodies? J Clin Invest 98:2235–2243
21. Torres M, May R, Scharff MD, Casadevall A (2005) Variable-region-identical antibodies differing in isotype demonstrate differences in fine specificity and idiotype. J Immunol 174:2132–2142
22. Wandstrat A, Wakeland E (2001) The genetics of complex autoimmune diseases: non-MHC susceptibility genes. Nat Immunol 2:802–809
23. Magira EE, Papaioakim M, Nachamkin I, Asbury AK, Li CY, Ho TW, Griffin JW, McKhann GM, Monos DS (2003) Differential distribution of HLA-DQ beta/DR beta epitopes in the two forms of Guillain–Barré syndrome, acute motor axonal neuropathy and acute inflammatory demyelinating polyneuropathy (AIDP): identification of DQ beta epitopes associated with susceptibility to and protection from AIDP. J Immunol 170:3074–3080

Campylobacter gene polymorphism as a determinant of clinical features of Guillain–Barré syndrome

M. Koga, MD, PhD; M. Takahashi, DVM, PhD; M. Masuda, MD, PhD; K. Hirata, MD, PhD; and N. Yuki, MD, PhD

Abstract—*Background:* Ganglioside epitopes on *Campylobacter jejuni* are hypothesized as the key to the development and characterization of Guillain–Barré syndrome (GBS), but a comprehensive theory has yet to be established. A *C jejuni* gene, *cst-II*, involved in the biosynthesis of ganglioside-like lipo-oligosaccharide, shows a polymorphism (Asn/Thr51) that affects ganglioside epitopes. *Objective:* To examine the hypothesis that this polymorphism determines autoantibody reactivity, and thereby neurologic presentations in GBS. *Methods: C jejuni* isolates were collected from 105 GBS (including its variants) and 65 uncomplicated enteritis patients. The authors examined the frequency of *cst-II* and polymorphism (Asn/Thr51) in connection with the bacterial ganglioside epitopes, autoantibody reactivities against GM1, GD1a, and GQ1b, and patients' neurologic findings. *Results:* Neuropathic strains more frequently had *cst-II*, in particular *cst-II* (Thr51), than did enteritic ones (85% vs 52%; $p < 0.001$). Strains with *cst-II* (Asn51) regularly expressed the GQ1b epitope (83%), whereas those with *cst-II* (Thr51) had the GM1 (92%) and GD1a (91%) epitopes. The presence of these bacterial epitopes in neuropathy patients corresponded to autoantibody reactivity. Patients infected with *C jejuni* (Asn51) more often were positive for anti-GQ1b IgG (56% vs 8%; $p < 0.001$) and had ophthalmoparesis (64% vs 13%; $p < 0.001$) and ataxia (42% vs 11%; $p = 0.001$). Patients who had *C jejuni* (Thr51) more frequently were positive for anti-GM1 (88% vs 35%; $p < 0.001$) and anti-GD1a IgG (52% vs 24%; $p = 0.006$) and had limb weakness (98% vs 71%; $p < 0.001$). *Conclusions:* The genetic polymorphism of *C jejuni* determines autoantibody reactivity as well as the clinical presentation of Guillain–Barré syndrome (GBS), possibly through modification of the host-mimicking molecule. The GBS paradigm is the first to explain the detailed pathogenesis of a postinfectious, autoimmune-mediated, molecular mimicry-triggering disorder.

NEUROLOGY 2005;65:1376–1381

Guillain–Barré syndrome (GBS) is characterized by limb weakness and loss of tendon reflexes, but has a variety of other neurologic presentations.[1] Anti-GM1 and anti-GD1a IgG antibodies are associated with axonal GBS,[2] whereas anti-GQ1b IgG antibody is specific to patients with Fisher syndrome (FS) or GBS with ophthalmoplegia.[3] *Campylobacter jejuni* is the most frequent antecedent infectious agent in GBS.[4] The critical factor that causes the development of neuropathy after *C jejuni* infection is unknown, but the bacterial lipo-oligosaccharide (LOS) is a candidate because its terminal sugar regions mimic the sugar residues of the gangliosides GM1, GD1a, and GQ1b.[5-7] The development of axonal GBS model after inoculation of rabbits with the GM1-like LOS has provided conclusive evidence for the hypothesis that the ganglioside-mimicry of *C jejuni* LOS is a cause of GBS.[8] However, determinant factor of anti-ganglioside antibody specificity and neurologic presentation in GBS remains unclear.

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

Determination of the complete genome sequence of *C jejuni* NCTC 11168 showed that many LOS biosynthesis genes are encoded in a large cluster.[9] A subsequent study identified the genes involved in the

Editorial, see page 1350

This article was previously published in electronic format as an Expedited E-Pub on September 14, 2005, at www.neurology.org.

From the Departments of Neurology (Drs. Koga, Hirata, and Yuki) and Microbiology (Dr. Masuda), Dokkyo University School of Medicine, Tochigi; and Department of Microbiology (Dr. Takahashi), Tokyo Metropolitan Institute of Public Health, Japan.

Supported in part by grants from the Ichiro Kanehara Foundation to M.K.; the Kanae Foundation for Life & Socio-Medical Science to M.K.; the Japan Intractable Diseases Research Foundation to M.K.; Mizutani Foundation for Glycoscience to N.Y.; a grant for Scientific Research (B) (KAKENHI 14370210 to N.Y.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; a Research Grant for Neuroimmunological Diseases to N.Y. from the Ministry of Health, Labour and Welfare of Japan; a Health Sciences Research Grant (Research on Psychiatric and Neurological Diseases and Mental Health) to N.Y. from the Ministry of Health, Labour, and Welfare of Japan; and a grant from the Human Frontier Science Program (RGP0038/2003-C to N.Y.).

Disclosure: The authors report no conflicts of interest.

Received April 4, 2005. Accepted in final form June 20, 2005.

Address correspondence and reprint requests to Dr. M. Koga, Department of Neurology, Dokkyo University School of Medicine, Kitakobayashi 880, Mibu, Shimotsuga, Tochigi 321-0293, Japan; e-mail: kogamrk@dokkyomed.ac.jp

transfer of galactose, *N*-acetylgalactosamine, and sialic acid to the LOS outer core.[10] Because ganglioside classification is based on the sialylation type (see figure E-1 on the *Neurology* Web site at www.neurology.org),[11] sialyltransferase-encoding genes may be associated with the variation in the ganglioside epitope on LOS. It was reported that *cst-II*, a gene encoding sialyltransferase,[10,12] was present in all eight strains with GQ1b-like LOS and proposed that its presence is associated with various ganglioside-like LOSs, although *cst-II* frequency did not differ between the GBS/FS and uncomplicated enteritis strains studied.[13] In contrast, based on tests of 28 GBS isolates, it was reported that the *cst-II* gene is more often present in GBS isolates than in enteritis isolates.[14] Whether the presence of this gene is a risk factor for developing neuropathy after *C jejuni* enteritis has yet to be proved.

The ganglioside-like structure of the *C jejuni* LOS is, in part, determined by the multiple mechanisms the bacterium uses to turn on or off a gene or to modulate the substrate specificities of its glycosyltransferases, as well as by different gene contents.[15] Interestingly, variation in the nucleotide sequence of *cst-II* might affect enzymatic activity; Cst-II (Thr51) has only α-2,3-sialyltransferase activity (monofunctional), whereas Cst-II (Asn51) has both α-2,3- and α-2,8-sialyltransferase activities (bifunctional).[15] Because both α-2,3- and α-2,8-sialyltransferase activities are required for the biosynthesis of GQ1b mimics such as GT1a- or GD1c-like LOS,[6,7] *cst-II* polymorphism is assumed to affect autoantibody reactivities through change in the ganglioside epitope on the LOS outer core, resulting in the diverse neurologic features shown by patients with GBS. In this study we used 105 *C jejuni* isolates from patients with GBS or a clinical variant and compared *cst-II* gene frequency in the neuropathic and enteritic strains. We also examined the hypothesis that the genetic polymorphism of the bacterium produces the differences in the clinical manifestation of GBS.

Methods. *Bacterial strains and patients.* Since 1990, we have received more than 8,000 requests from Japanese physicians to test serum anti-ganglioside antibodies from patients presenting with various neurologic disorders. On receipt of serum samples from patients with GBS or a clinical variant, we request the primary physicians to do a stool culture and to send the patient's stool specimen to the Tokyo Metropolitan Institute of Public Health for *C jejuni* isolation. From 1990 to 2002, 81 *C jejuni* strains were isolated successfully by one of the authors (M.T.), and 24 strains were obtained from hospitals. These were used in this study (n = 105: GBS, 80; FS, 20; Bickerstaff brainstem encephalitis,[16] 3; acute ophthalmoparesis,[17] 1; acute oropharyngeal palsy,[18] 1). Most of the strains used were included in our previous study.[19] Two, OH4384 and OH4382, were obtained from sibling GBS patients[6,20]; the others were from sporadic cases with geographically equal distributions. A questionnaire was filled in by the primary physician as to whether diagnostic criteria for GBS or a clinical variant were fulfilled. We also reviewed the patients' medical records to ascertain diagnoses and neurologic findings. GBS, FS, Bickerstaff brainstem encephalitis, and acute ophthalmoparesis diagnoses were all based on clinical criteria.[21,22] ELISAs were used to test for the presence of IgG antibodies to GM1, GD1a, and GQ1b, as reported elsewhere.[23] Sixty-five strains that had been isolated from patients with uncomplicated enteritis and collected throughout Japan were the controls. Anti-ganglioside antibodies were not tested in the enteritis patients because previous studies showed that such patients did not have the autoantibodies.[24,25]

Detection of ganglioside epitopes on LOS. Crude LOS fractions were prepared from the isolates, as described elsewhere,[26] with minor modifications.[27] We first performed sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and silver staining to ensure LOS had been extracted then used western blotting with the cholera toxin B-subunit (a ligand for GM1-oligosaccharide structure) to examine whether the GM1 epitope was present on it. A 5 μL portion of the bacterial lysate was added to each well, after which the samples were separated on 15% tricine-SDS-polyacrylamide gels (SPU-15S series, Atto Corporation, Tokyo, Japan).[28] After electrophoresis, the LOS samples were blotted on polyvinylidene difluoride membranes (Atto Corporation), and the membranes incubated at 4 °C for 2 hours with the peroxidase-conjugated cholera toxin B-subunit (List Biologic Laboratories, Campbell, CA) diluted 1:2,000 in phosphate-buffered saline containing 0.5% casein. Binding was made visible with 4-chloro-1-naphtol (Konica Immunostaining HRP-1000, Konica, Tokyo, Japan).

Ganglioside-like LOS also was investigated by thin-layer chromatography (TLC) with immunostaining because this method decreases the volume of reagent needed in the immunostaining step. The reagents used are the monoclonal antibodies (GB1 [anti-GD1a], GB2 [anti-GM1], and FS1 [anti-GQ1b]),[8,27] and sera from patients with GBS (S6960 [anti-GM1] and S5174 [anti-GD1a]) or FS (S7577 [anti-GQ1b]). A 10 μL portion of each bacterial lysate was spotted on a precoated Silica Gel 60 TLC plate (Merck, Darmstadt, Germany), developed with an *n*-propanol-water-25% ammonia solution (6:5:1, by volume), after which the plates were immunostained as reported elsewhere.[23] The TLC analysis was done by one of the authors (M.K.).

PCR and DNA sequencing. A single bacterial colony was suspended in 300 μL of sterile deionized distilled water and boiled for 10 minutes. After centrifugation at 15,000 rpm for 1 minute, the supernatant was used as the template in the PCR amplification. Oligonucleotide primers were designed based on the reported *C jejuni cst-II* sequence (GenBank accession no. AF400048): forward directional primer, 17F (5′-TTTCTGGAAATGGACCAAGTTT-3′), and reverse, 220R (5′-CGGTCTCATATTCTTGATTTTGG-3′). These primers amplify the 204 bp fragment of the *cst-II* gene carrying the 51st codon. Amplification reactions were run in a total volume of 50 μL with 20 pmol of each primer, 10 μL of template, and 2.5 units of *Taq* DNA polymerase (TaKaRa Ex Taq, Takara Bio Inc., Shiga, Japan), 40 nmol dNTPs, and buffer (2 mM Mg^{2+}), as recommended by the manufacturer. After a denaturation step of 5 minutes at 95 °C, the amplification mixture underwent 30 cycles of amplification, each cycle consisting of 10 seconds at 94 °C, 10 seconds at 55 °C, and 30 seconds at 72 °C, in a GeneAmp PCR System 9700 (PE Biosystems, Foster City, CA). PCR products were purified with a QIAquick PCR Purification Kit (Qiagen K.K., Tokyo, Japan). Nucleotide sequences of PCR products were determined with an ABI BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Statistical analysis. Frequency differences between the groups were compared by means of Fisher's exact test using SPSS 12.0J software (SPSS Inc., Chicago, IL). A difference was considered significant when the two-sided p value was less than 0.05.

Results. cst-II *Gene frequency and polymorphism (Thr/Asn51).* PCR and direct nucleotide sequence analyses showed that the *cst-II* gene more often was present in neuropathic than enteritic strains (table 1). Although the 51st codon of *cst-II* is reported to be AAT (corresponding to Asn) or ACC (Thr),[15] some strains in our study had AAC (Asn). This indicates that a single base change (A to C or C to A) which may occur during infection can alter the substrate specificity of Cst-II (monofunctional or bifunctional). *cst-II* (Thr51) was more common in the neuropathic (50%) than enteritic (25%) strains ($p = 0.001$), whereas *cst-II* (Asn51) did not differ (34% vs 26%; $p = 0.31$). Also, 9 of 16

Table 1 *Comparison of genetic and phenotypic properties of neuropathic and enteritic* Campylobacter jejuni *strains*

	Neuropathic strains, n = 105	Enteritic strains, n = 65	*p* Value
Gene			
cst-II	89 (85)	33 (51)	<0.001
cst-III	9 (9)	15 (23)	0.012
GM1 epitope*			
Cholera toxin	62 (59)	25 (38)	0.01
GB2	63 (60)	25 (38)	0.007
S6960	60 (57)	22 (34)	0.004
GD1a epitope*			
GB1	51 (49)	13 (20)	<0.001
S5174	49 (47)	12 (18)	<0.001
GQ1b epitope*			
FS1	22 (21)	13 (20)	1.0
S7577	32 (30)	17 (26)	0.60

Values are n (%).

* The reagents used are the monoclonal antibodies (GB1 [anti-GD1a], GB2 [anti-GM1], and FS1 [anti-GQ1b]), and sera from patients with Guillain-Barré syndrome (S6960 [anti-GM1] and S5174 [anti-GD1a]) or Fisher syndrome (S7577 [anti-GQ1b]). Cholera toxin (B-subunit) is a ligand for GM1-oligosaccharide structure.

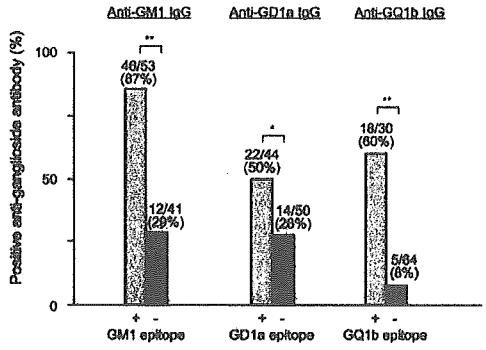


Figure. The ganglioside-like lipo-oligosaccharide of Campylobacter jejuni *and anti-ganglioside autoantibody reactivity in patients with Guillain–Barré syndrome or a variant. Frequencies of positive IgG antibodies against GM1 (left), GD1a (middle), and GQ1b (right) are compared between patients whose isolates carry ganglioside epitope on lipo-oligosaccharide (LOS) (pale bars) or not (dark bars). *p = 0.04; **p < 0.001.*

neuropathic strains without *cst-II* had the *cst-III* gene,[12] and this gene was significantly less frequent in the neuropathic strains compared to the enteritic ones (see table 1).

Ganglioside-like LOS. Although we used two or three reagents in the detection of each ganglioside epitope, the overall results were identical, except for some discrepancies probably due to differences in the sensitivities of the reagents (see table 1). Figure E-2 shows the ganglioside epitope detection in representative strains. Neuropathic strains more commonly expressed GM1 and GD1a epitopes than did enteritic strains. In contrast, the frequency of the GQ1b epitope did not differ between them. Immunostaining results for the patients' sera showed that the GM1 and GD1a epitopes commonly were present in the same neuropathic strains (n = 48; 46%), whereas the GQ1b epitope was present isolatedly (n = 27; 26%). GQ1b and GM1 epitopes coexisted in five strains. Seventeen (16%) of the strains had none of the ganglioside epitopes examined.

cst-II Polymorphism (Thr/Asn51) was closely related to the ganglioside epitopes on LOS in both the neuropathic and enteritic strains. Asn51 strains more often expressed GQ1b epitope than did the others (table 2). In contrast, most Thr51 strains carried GM1- and GD1a-like LOS. Because the 53rd codon of *cst-II* is reported to affect α-2,3- and α-2,8-sialyltransferase activities in an Asn51 strain,[15] we analyzed its association with ganglioside-like LOS. Of the seven strains with *cst-II* (Asn51/Ile53) thought to have relatively low α-2,3- and α-2,8-sialyltransferase activities,[15] four had both GQ1b and GM1 epitopes, three had none. In contrast, all 22 strains (Asn51/Ser53 and Asn51/Gly53) thought to have high α-2,8-sialyltransferase activity had only the GQ1b epitope. Four of seven strains (Asn51/Tyr53) also had only the GQ1b epitope. The other three had neither epitope.

Serologic and neurologic features. Detailed neurologic information was available for 97 neuropathy patients, and pretreatment serum samples were obtained within 4 weeks of neuropathic onset for 94. Serum anti-ganglioside autoantibody reactivities overall corresponded to the ganglioside epitopes on the LOSs of the isolates (figure): GM1 epitope on LOS and anti-GM1 IgG (*p* value < 0.001);

Table 2 *Association of bacterial properties with* cst-II *content and polymorphism in neuropathic* Campylobacter jejuni *strains*

	cst-II			*cst-II* (Asn51)			*cst-II* (Thr51)		
	Present	Absent	*p* Value	Present	Absent	*p* Value	Present	Absent	*p* Value
Lipo-oligosaccharide	n = 89	n = 16		n = 36	n = 69		n = 53	n = 52	
GM1 epitope*	53 (60)	7 (44)	0.28	4 (11)	56 (81)	<0.001	49 (92)	11 (21)	<0.001
GD1a epitope*	48 (54)	1 (6)	<0.001	0	49 (71)	<0.001	48 (91)	1 (2)	<0.001
GQ1b epitope*	31 (35)	1 (6)	0.04	30 (83)	2 (3)	<0.001	1 (2)	31 (60)	<0.001

Values are n (%).

* Judged by results of thin-layer chromatography with immunostaining of patients' sera (S6960 [anti-GM1], S5174 [anti-GD1a], and S7577 [anti-GQ1b]) having high anti-ganglioside antibody titer.

Table 3 *Association of patients' clinical features with* cst-II *content and polymorphism of their* Campylobacter jejuni *isolates*

	cst-II			*cst-II* (Asn51)			*cst-II* (Thr51)		
	Present	Absent	*p* Value	Present	Absent	*p* Value	Present	Absent	*p* Value
IgG antibody to	n = 80	n = 14		n = 32	n = 62)		n = 48	n = 46	
GM1	49 (61)	9 (64)	1.0	7 (22)	51 (82)	<0.001	42 (88)	16 (35)	<0.001
GD1a	32 (40)	4 (29)	0.56	7 (22)	29 (47)	0.03	25 (52)	11 (24)	0.006
GQ1b	20 (25)	3 (21)	1.0	18 (56)	5 (8)	<0.001	2 (4)	21 (46)	<0.001
Neurological sign	n = 81	n = 16		n = 33	n = 64		n = 48	n = 49	
Ophthalmoparesis	25 (31)	4 (25)	0.77	21 (64)	8 (13)	<0.001	4 (8)	25 (51)	<0.001
Facial palsy	14 (17)	1 (6)	0.45	11 (33)	4 (6)	0.001	3 (6)	12 (24)	0.02
Bulbar palsy	17 (21)	0	0.07	13 (39)	4 (6)	<0.001	4 (8)	13 (27)	0.03
Limb weakness	69 (85)	13 (81)	0.71	22 (67)	60 (94)	0.001	47 (98)	35 (71)	<0.001
Ataxia	17 (21)	4 (25)	0.74	14 (42)	7 (11)	0.001	3 (6)	18 (37)	<0.001
Sensory disturbance	26 (32)	3 (19)	0.38	9 (27)	20 (31)	0.82	17 (35)	12 (24)	0.27
Diagnosis	n = 89	n = 16		n = 36	n = 69		n = 53	n = 52)	
Guillain–Barré syndrome	68 (76)	12 (75)	1.0	17 (47)	63 (91)	<0.001	51 (96)	29 (56)	<0.001
Fisher syndrome	16 (18)	4 (25)	0.50	14 (39)	6 (9)	<0.001	2 (4)	18 (35)	<0.001

Values are n (%).

GD1a epitope and anti-GD1a IgG (p value = 0.04); and GQ1b epitope and anti-GQ1b IgG ($p < 0.001$).

As expected, *cst-II* polymorphism was closely related to serum anti-ganglioside autoantibody reactivities, and therefore to neurologic features, whereas gene content was not (table 3). Patients from whom *C jejuni* (Asn51) had been isolated more often had serum anti-GQ1b IgG autoantibody, cranial nerve palsies, and ataxia. In contrast, *C jejuni* (Thr51) was associated with anti-GM1 and anti-GD1a IgG antibodies and limb weakness. The diagnosis was GBS for most patients with *C jejuni* (Thr51), whereas it was GBS for 47% and FS for 39% of patients with *C jejuni* (Asn51). Conversely, compared to the enteritis patients, FS patients more often had been infected by *C jejuni* (Asn51) (70% vs 26%; p value = 0.001), whereas GBS patients had been by *C jejuni* (Thr51) (64% vs 25%; p value < 0.001). Variation in the 53rd codon was not associated with any type of autoantibody or neurologic feature in spite of its association with ganglioside-like LOS (data not shown).

Discussion. The pathogenesis of many post-infectious disorders is still unknown, no autoantigens or virulence factors having been identified. For example, why some group A streptococci can cause acute rheumatic fever and others acute glomerulonephritis is unclear.[29] In contrast, we are the first to show the detailed molecular mechanism of GBS after *C jejuni* enteritis, based on our and others findings that the genetic polymorphism of the bacterium alters the substrate specificity of the LOS biosynthesis enzyme[15] and that autoantibody reactivity determines the clinical presentation of GBS.[30] These findings suggest that the genetic polymorphism of antecedent agents determines autoantibody reactivities and clinical manifestations through change to the host-mimicking molecule in some post-infectious disorders. We believe that GBS is the first paradigm to explain the detailed pathogenesis of a post-infectious, autoimmune-mediated, molecular mimicry-triggering disorder.

We confirmed results of a previous report that *cst-II* more frequently exists in GBS than in enteritis isolates, indicative that its presence is a risk factor for developing GBS.[14] However, considerable numbers of strains from patients with uncomplicated enteritis also had *cst-II* gene, indicating that *cst-II* gene is necessary but not adequate for initiating autoimmune response, although *cst-II* genotype is important in determining antibody reactivity when autoimmune response is triggered. Certain other genes (e.g., *cst-III*[12] or an unidentified gene) might produce the enzyme protein instead, subsequently sialylating LOS. Three genes, *cst-I*, *-II*, and *-III*, are reported to encode sialyltransferase protein,[12,15] but *cst-I* was lacking in some strains with sialylated LOS and therefore is unlikely to be responsible for LOS sialylation.[10] Because Cst-III appears to have only α-2,3-sialyltransferase activity (monofunction),[15] *cst-II* content must be essential for α-2,8-sialyltransferase activity and thereby biosynthesis of the GQ1b epitope.[13,15]

Most of the previous studies have failed to find a specific *C jejuni* genotype for GBS and FS.[31-35] It was recently reported that the class A LOS biosynthesis locus was over-represented in GBS-associated as compared to enteritis strains, whereas all four of the FS-related strains belonged to class B.[36] The authors suspected that the frequent expression of a GM1 epitope in class A and a GQ1b epitope in class B strains is responsible for the development of GBS and FS. Their findings, however, do not provide the answers as to which difference leads to diverse

ganglioside-mimics (GM1 and GQ1b) in spite of there being almost the same class A and B gene profiles.[15] In contrast, our data clearly indicate that both presence and polymorphism of bacterial *cst-II* have a major role in the type of ganglioside-like structure on LOS, thereby determining autoantibody reactivity and the pattern of neurologic presentation. Phase variation owing to a homopolymeric G-tract in *cst-II* or other LOS synthesis genes also may be related to what ganglioside epitopes are present.[10] Along with those of a previous study,[15] however, our findings suggest that mainly it is variation in the *cst-II* 51st codon and supplemental variation in the 53rd codon that determine which ganglioside epitopes are present on LOS, but only the former was related to the autoantibody reactivity in and neurologic features of neuropathy patients. Interestingly, *Haemophilus influenzae*, a pathogen recently suggested to cause GBS and FS,[27,37] also has sialylated LOS.[38] Three genes (*lic3A*, *siaA*, *lsgB*) have been cloned for that sialylation enzyme.[39,40] Whether the polymorphism of *H influenzae* genes also is related to autoantibody reactivity and consequently to the neurologic features of GBS and FS requires investigation.

C jejuni strains which had been isolated from anti-GQ1b antibody-positive patients often carried GQ1b epitope on the LOS.[41] The specificity of anti-ganglioside antibody induced by immunization of *C jejuni* LOS overall corresponds to ganglioside epitopes on the LOS in rabbits.[42] In this study, we showed that the target ganglioside (GM1, GD1a, or GQ1b) for serum autoantibody corresponds to the LOS-mimicking gangliosides of isolates from individual patients. However, other gangliosides such as GM1b and GalNAc-GD1a could be target antigens for autoantibodies in GBS[30] and further investigation is necessary to explain the variety of the clinical manifestation of GBS in more detail.

Acknowledgment

The authors thank Dr. Michel Gilbert (Institute for Biologic Sciences, National Research Council of Canada, Ottawa, Canada) for critical reading of the paper; Saiko Koike and Takashi Namatame (Institute for Medical Science, Dokkyo University School of Medicine, Tochigi, Japan) for help with the bacterial and sequence analyses; Maki Okazaki (Department of Neurology, Dokkyo University School of Medicine, Tochigi, Japan) for technical assistance; and Dr. Yukihiro Nishimoto (Department of Pediatrics, Kinan General Hospital, Wakayama, Japan) and Dr. Shigeru Matsushita (Tama Branch Laboratory, Tokyo Metropolitan Institute of Public Health, Tokyo, Japan) for providing the *C jejuni* strains from enteritis patients.

References

1. Ropper AH. The Guillain-Barré syndrome. N Engl J Med 1992;326: 1130–1136.
2. Ogawara K, Kuwabara S, Mori M, et al. Axonal Guillain-Barré syndrome: relation to anti-ganglioside antibodies and *Campylobacter jejuni* infection in Japan. Ann Neurol 2000;48:624–631.
3. Chiba A, Kusunoki S, Obata H, et al. Serum anti-GQ1b IgG antibody is associated with ophthalmoplegia in Miller Fisher syndrome and Guillain-Barré syndrome: clinical and immunohistochemical studies. Neurology 1993;43:1911–1917.
4. Jacobs BC, Rothbarth PH, van der Meché FGA, et al. The spectrum of antecedent infections in Guillain-Barré syndrome: a case-control study. Neurology 1998;51:1110–1115.
5. Yuki N, Taki T, Inagaki F, et al. A bacterium lipopolysaccharide that elicits Guillain-Barré syndrome has a GM1 ganglioside-like structure. J Exp Med 1993;178:1771–1775.
6. Aspinall GO, McDonald AG, Pang H, et al. Lipopolysaccharides of *Campylobacter jejuni* serotype O:19 structures of core oligosaccharide regions from the serostrain and two bacterial isolates from patients with Guillain-Barré syndrome. Biochemistry 1994;33:241–249.
7. Shin JE, Sckloo S, Mainkar, et al. Lipo-oligosaccharides of *Campylobacter jejuni* serotype O:10: structures of core oligosaccharide regions from a bacterial isolate from a patient with the Miller-Fisher [sic] syndrome and from the serotype reference strain. Carbohydr Res 1998; 305:223–232.
8. Yuki N, Susuki K, Koga M, et al. Carbohydrate mimicry between human ganglioside GM1 and *Campylobacter jejuni* lipo-oligosaccharide causes Guillain-Barré syndrome. Proc Natl Acad Sci USA 2004;101: 11404–11409.
9. Parkhill J, Wren BW, Mungall K, et al. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature 2000;403:665–668.
10. Gilbert M, Brisson J-R, Karwaski M-F, et al. Biosynthesis of ganglioside mimics in *Campylobacter jejuni* OH4384: identification of the glycosyltransferase genes, enzymatic synthesis of model compounds, and characterization of nanomole amounts by 600-MHz ^{1}H and ^{13}C NMR analysis. J Biol Chem 2000; 275: 3896–3906.
11. Svennerholm L. Chromatographic separation of human brain gangliosides. J Neurochem 1963;10:613–623.
12. Guerry P, Ewing CP, Hickey TE, et al. Sialylation of lipooligosaccharide cores affects immunogenicity and serum resistance of *Campylobacter jejuni*. Infect Immun 2000;68:6656–6662.
13. van Belkum A, van den Braak N, Godschalk P. *Campylobacter jejuni* gene associated with immune-mediated neuropathy. Nat Med 2001;7: 752–753.
14. Nachamkin I, Liu J, Li M, et al. *Campylobacter jejuni* from patients with Guillain-Barré syndrome preferentially expresses a GD1a-like epitope. Infect Immun 2002;70:5299–5303.
15. Gilbert M, Karwaski M-F, Bernatchez S, et al. The genetic bases for the variation in the lipo-oligosaccharide of the mucosal pathogen, *Campylobacter jejuni*: biosynthesis of sialylated ganglioside mimics in the core oligosaccharide. J Biol Chem 2002;277:327–337.
16. Odaka M, Yuki N, Yamada M, et al. Bickerstaff's brainstem encephalitis: clinical features of 62 cases and a subgroup associated with Guillain-Barré syndrome. Brain 2003;126:2279–2290.
17. Yuki N, Odaka M, Hirata K. Acute ophthalmoparesis (without ataxia) associated with anti-GQ1b IgG antibody: clinical features. Ophthalmology 2001;108:196–200.
18. O'Leary CP, Veitch J, Durward WF, et al. Acute oropharyngeal palsy is associated with antibodies to GQ1b and GT1a gangliosides. J Neurol Neurosurg Psychiatry 1996;61:649–651.
19. Takahashi M, Koga M, Yokoyama K, Yuki N. Epidemiology of *Campylobacter jejuni* isolated from patients with Guillain-Barré and Fisher syndromes in Japan. J Clin Microbiol 2005;43:335–339.
20. Yuki N, Tsujino Y. Familial Guillain-Barré syndrome subsequent to *Campylobacter jejuni* enteritis. J Pediatr 1995;126:162.
21. Asbury AK, Cornblath DR. Assessment of current diagnostic criteria for Guillain-Barré syndrome. Ann Neurol 1990;27(suppl):S21–24.
22. Odaka M, Yuki N, Hirata K. Anti-GQ1b IgG antibody syndrome: clinical and immunological range. J Neurol Neurosurg Psychiatry 2001;70: 50–55.
23. Yuki N, Tagawa Y, Irie F, et al. Close association of Guillain-Barré syndrome with antibodies to minor monosialogangliosides GM1b and GM1α. J Neuroimmunol 1997;74:30–34.
24. Yuki N, Yoshino H, Sato S, Miyatake T. Acute axonal polyneuropathy associated with anti-GM1 antibodies following *Campylobacter* enteritis. Neurology 1990;40:1900–1902.
25. Rees JH, Gregson NA, Hughes RAC. Anti-ganglioside GM1 antibodies in Guillain-Barré syndrome and their relationship to *Campylobacter jejuni* infection. Ann Neurol 1995;38:809–816.
26. Hitchcock PJ, Brown TM. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J Bacteriol 1983;154:269–277.
27. Koga M, Gilbert M, Li J, et al. Antecedent infections in Fisher syndrome. A common pathogenesis of molecular mimicry. Neurology 2005; 64:1605–1611.
28. Schägger H, von Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to100 kDa. Anal Biochem 1987;166:368–379.
29. Stollerman GH. Rheumatic fever. Lancet 1997;349:935–942.
30. Willison HJ, Yuki N. Peripheral neuropathies and anti-glycolipid antibodies. Brain 2002;125:2591–2625.
31. Endtz HP, Ang CW, van den Braak N, et al. Molecular characterization of *Campylobacter jejuni* from patients with Guillain-Barré and Miller Fisher syndromes. J Clin Microbiol 2000;38:2297–2301.
32. Duim B, Ang CW, van Belkum A, et al. Amplified fragment length polymorphism analysis of *Campylobacter jejuni* strains isolated from chickens and from patients with gastroenteritis or Guillain-Barré or Miller Fisher syndromes. Appl Environ Microbiol 2000;66:3917–3923.