**Haemophilus influenzae** infection and Guillain–Barré syndrome

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**Summary**

It has been reported recently that *Haemophilus influenzae* can elicit an axonal form of Guillain–Barré syndrome. To investigate the incidence and features of *H. influenzae*-related Guillain–Barré syndrome, anti-*H. influenzae* antibodies were measured by enzyme-linked immunosorbent assay (ELISA) in 46 consecutive Japanese patients with Guillain–Barré syndrome, 49 normal controls, 24 patients with multiple sclerosis and 27 patients with amyotrophic lateral sclerosis (ALS). Whole bacteria of non-encapsulated (non-typable) *H. influenzae* isolated from one of the Guillain–Barré syndrome patients was the antigen used. Elevated anti-*H. influenzae* antibodies for two or three classes of IgG, IgM and IgA were found in six (13%) Guillain–Barré syndrome patients, but not in the normal controls and patients with multiple sclerosis or ALS. The incidence was significantly higher in patients with Guillain–Barré syndrome than in the normal controls ($P = 0.009$). Western blot analysis confirmed that the *H. influenzae*-positive patients’ IgG recognized the lipopolysaccharides of *H. influenzae*. Guillain–Barré syndrome patients with anti-*H. influenzae* antibodies showed relatively uniform clinical and laboratory features: prodromal respiratory infection, less frequent cranial and sensory nerve involvement, pure motor axonal degeneration on electrophysiology, and positivity for IgG anti-GM1 antibodies. Although the features were similar to those in Guillain–Barré syndrome patients infected by *Campylobacter jejuni*, the recoveries seemed to be better in patients with *H. influenzae*-related Guillain–Barré syndrome. It is concluded that a form of Guillain–Barré syndrome occurs after respiratory infection by *H. influenzae* in the Japanese population. A particular strain of non-typable *H. influenzae* has a ganglioside GM1-like structure and elicits axonal Guillain–Barré syndrome similar to *C. jejuni*-related Guillain–Barré syndrome.

**Keywords:** Guillain–Barré syndrome; *Haemophilus influenzae*; anti-GM1 antibody; axonal degeneration; acute motor axonal neuropathy

**Abbreviations:** AIDP = acute inflammatory demyelinating polyneuropathy; ALS = amyotrophic lateral sclerosis; AMAN = acute motor axonal neuropathy; CMV = cytomegalovirus; EBV = Epstein–Barr virus; ELISA = enzyme-linked immunosorbent assay; LPS = lipopolysaccharide; OD = optical density

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**Introduction**

Guillain–Barré syndrome, an acute inflammatory, predominantly motor polyneuropathy (Asbury and Cornblath, 1990), is preceded by an acute infectious illness in approximately two-thirds of cases, the onset of neuropathic symptoms being preceded by respiratory infection in 30–45% of cases and by gastrointestinal infection in 15–20% of cases (Winer et al., 1988; Boucquey et al., 1991). Various infectious agents are reported as possibly having an association with Guillain–Barré syndrome (Winer et al., 1988; Ropper et al., 1991), but the causal relationship between the infectious agent for prodromal illness and peripheral nerve damage is not known. *Campylobacter jejuni* was recently identified as the most common cause of preceding infection in Guillain–Barré syndrome (Kaldor and Speed, 1984; Winer et al., 1988; Mishu and Blaser, 1993; Rees et al., 1995b), and *C. jejuni*-related Guillain–Barré syndrome is speculated to be associated with axonal degeneration of the motor nerves (Yuki et al., 1990; Rees et al., 1995a; Visser et al., 1995) or positivity for anti-ganglioside GM1 antibody (Rees et al., 1995a; Jacobs et al., 1996). Molecular mimicry between the GM1 in neural tissue and the GM1-like structure on the lipopolysaccharide (LPS) of *C. jejuni* has also been suggested (Yuki et al., 1992a, 1993; Oomes et al., 1995). The majority of Guillain–Barré syndrome...
patients, however, have had prodromal respiratory infection. Cytomegalovirus (CMV), Epstein–Barr virus (EBV) and *Mycoplasma pneumoniae* are also possible causes of respiratory tract infections that precede Guillain–Barré syndrome, but their frequencies are <15% (Winer *et al.*, 1988; Boucquey *et al.*, 1991; Hao *et al.*, 1998; Jacobs *et al.*, 1998).

We reported recently a patient with Guillain–Barré syndrome subsequent to respiratory infection by *Haemophilus influenzae* and the possible presence of a GM1-like structure on the surface of this bacterium (Mori *et al.*, 1999). To investigate the incidence and the clinical and laboratory features of *H. influenzae*-related Guillain–Barré syndrome, we carried out a retrospective case–control study of 46 Guillain–Barré syndrome patients in whom anti-*H. influenzae* antibodies had been detected.

**Methods**

**Subjects**

Forty-six consecutive Guillain–Barré syndrome patients seen at Chiba University Hospital or its affiliated hospitals between January 1989 and June 1998 were studied. All fulfilled the clinical criteria for Guillain–Barré syndrome (Asbury and Comblath, 1990). Clinical disabilities were evaluated by Hughes functional grading (Hughes *et al.*, 1978), and patients were followed for up to 3 months after onset. Serum samples obtained within 4 weeks of the onset of neurological symptoms were stored at −80°C until used. Whenever possible, sputum or stool samples were collected from each patient. Serum samples from 49 normal controls and 51 patients with other neurological diseases seen during the same period, including 24 patients with multiple sclerosis and 27 with amyotrophic lateral sclerosis (ALS), who had not had any obvious infectious events during the 4 weeks before the collection of the blood sample, were the controls.

**Laboratory investigations**

**Anti-**-*H. influenzae* **antibody assay**

Serum anti-*H. influenzae* antibody titre was measured by the enzyme-linked immunosorbent assay (ELISA), as described previously (Kerttula *et al.*, 1987; Yuki *et al.*, 1992b; Burman *et al.*, 1994; Kurtti *et al.*, 1997), but with the following minor modifications. Strains of non-typable *H. influenzae* isolated from the sputum of one of the patients with Guillain–Barré syndrome (Mori *et al.*, 1999) enrolled in this study were used to prepare the *H. influenzae* antigen. They were grown on chocolate agar for 48 h at 37°C in 5% CO₂, inactivated in 1% formaldehyde, then washed. The product was used as the *H. influenzae* antigen. Wells of poly styrene plates were coated with the antigen in 100 µl carbonate buffer (50 mmol/l NaHCO₃, 50 mmol/l Na₂CO₃, pH 9.6) then washed with solution A (20 mmol/l Tris buffer, pH 7.4, 100 mmol/l NaCl, 0.5% skim milk, 0.05% Tween-20), after which they were blocked with 200 µl solution B (20 mmol/l Tris buffer, pH 7.4, 100 mmol/l NaCl, 3% skim milk, 0.05% Tween-20) overnight. The plates were then incubated overnight with 100 µl serum diluted 1 : 1000 in solution B, after which they were washed and incubated for 90 min at room temperature with peroxidase-conjugated goat anti-human IgG, IgM and IgA diluted 1 : 2000 in solution A. After another wash, the plates were developed with the substrate solution [containing 2.2′-azino-bis-(3-ethylbenzothiazolene sulphonate) (ABTS; Zymed, San Francisco, Calif., USA) and hydrogen peroxide in 0.1 M citrate buffer]. Optical density (OD) values at 405 nm were corrected by subtracting the OD obtained for each well without antigen. Serum was considered anti-*H. influenzae*-positive if its optical density was more than three standard deviations above the mean value for the 49 normal control samples. Each positive sample was diluted serially, starting at 1 : 1000. Antibody titre was defined as the highest serum dilution at which the OD was ≥0.1. The same assay was carried out twice to confirm the reproducibility of the results. Subjects were considered *H. influenzae*-positive if they had elevated anti-*H. influenzae* antibody for two or three classes of IgG, IgM or IgA.

**Western blot analysis**

Purified *H. influenzae* LPS (Westphal *et al.*, 1965) was prepared as described previously, from the non-typable strain used in the ELISA described above. *Escherichia coli* LPS was purchased from Difco Laboratories (Mich., USA). Sodium dodecyl sulphate–polyacrylamide gel electrophoresis was performed according to Laemmli (Laemmli, 1970); the separating gels contained 15% acrylamide and 2 M urea. All the preparations were transferred electrophoretically onto polyvinylidene difluoride sheets. Unreactive binding sites were blocked with blocking solution containing 3% skim milk in phosphate-buffered saline. The blots were incubated for 2 h at room temperature with patients’ sera diluted 1 : 200 in washing solution containing 0.2 M NaCl, 0.2% Triton X-100, and 0.05 M Tris–HCl, pH 7.5. The strips were washed, then incubated for 2 h at room temperature with peroxidase-conjugated antibodies to human IgG diluted 1 : 1000 in washing solution. After another wash, the reaction products were developed with enhanced chemiluminescence reagents (Amersham, San Francisco, Calif., USA) and exposed to film.

**Anti-ganglioside antibody assay**

IgG and IgM class antibodies to gangliosides GM2, GM1, GM1b, GD1a, GalNac-GD1a, GD1b, GQ1b and GT1b were measured by an ELISA described elsewhere (Koga *et al.*, 1998b) by one person (M. Koga, School of Medicine, Dokkyo University). Antibody titre was the highest serum dilution at which the optical density at 492 nm was ≥0.1. Serum was considered positive when the titre was ≥1 : 500.
H. influenzae and Guillain–Barré syndrome

Fig. 1 ELISA values for IgG anti-*H. influenzae* antibodies (A), IgM anti-*H. influenzae* antibodies (B) and IgA anti-*H. influenzae* antibodies (C). GBS = Guillain–Barré syndrome; NC = normal controls; OND = other neurological diseases (multiple sclerosis and ALS). Dashed lines show cut-off values (3 SD above the mean value for normal controls).

**Assays for anti-C. jejuni, anti-CMV and anti-M. pneumoniae antibodies**

IgG anti-*C. jejuni* antibody also was measured by an ELISA described elsewhere (Koga *et al.*, 1998b). IgM antibodies against CMV were measured with the ELISA. IgM antibodies against EBV-specific capsid antigen with the indirect fluorescent antibody test, and *M. pneumoniae* antibodies with a commercial particle agglutination and complement fixation test (Special Reference Laboratory, Tokyo, Japan).

**Electrodiagnostic studies**

Nerve conduction studies were done by the conventional procedure within 3 weeks of onset of neurological signs. Patients were classified as having acute motor axonal neuropathy (AMAN) (motor axonal Guillain–Barré syndrome) or acute inflammatory demyelinating polyneuropathy (AIDP) (demyelinating Guillain–Barré syndrome) using the electrodiagnostic criteria of Ho and colleagues (Ho *et al.*, 1995).

**Statistical analysis**

Differences in proportions were tested with Fisher’s exact test using StatView version 4.0 software.

**Results**

**Anti-*H. influenzae* antibody assay**

ELISA results of the IgG, IgM and IgA anti-*H. influenzae* antibody assays are shown in Fig. 1. Elevated anti-*H. influenzae* antibodies for two or three classes of IgG, IgM or IgA were present in six (13%) of the patients with Guillain–Barré syndrome, but not in the normal controls or patients with other neurological diseases (multiple sclerosis or ALS). In the Guillain–Barré syndrome patients, antibodies were classified as IgG in six cases, IgM in four, IgA in four, both IgG and IgM in four, and both IgG and IgA in two. No patient had both IgM and IgA. All the antibodies in the normal controls and in four patients with other neurological diseases were of the IgM class (one multiple sclerosis and three ALS patients). Positive serology for *H. influenzae* infection was significantly more frequent in patients with Guillain–Barré syndrome than in the normal controls (*P* = 0.01) and patients with other neurological diseases (*P* = 0.009). In patients with Guillain–Barré syndrome, IgG anti-*H. influenzae* antibody was positive more frequently than in normal controls (*P* = 0.02) and patients with other neurological diseases (*P* = 0.02). There were no significant differences in the frequencies of elevated IgM and IgA anti-*H. influenzae* antibodies among the three groups. Figure 2

Fig. 2 Longitudinal study of IgG anti-*H. influenzae* antibody titre in the anti-*H. influenzae*-positive Guillain–Barré syndrome patients. Data for five of the six patients are shown (the serum of the sixth patient was not sampled sequentially after 2 weeks).
patients had low compound muscle action potentials after distal stimulation (80% of normal lower limits) in multiple nerves, but no prolongation of distal latencies, decreases in conduction velocities, or temporal dispersion of responses. On electromyography 2 or 3 weeks after onset, there were amply fibrillation potentials in three of the four patients. In the other two patients who did not have the AMAN pattern, the absence of F waves was an isolated abnormality, and peripheral nerve conduction was entirely normal. Sensory nerve conduction was normal in all six patients. In all six patients, anti-ganglioside antibodies were present. Positivity for IgG anti-ganglioside antibodies was found for ganglioside GM1 (n = 5), GM1b (n = 3), GD1a (n = 4), GalNac-GD1a (n = 1), GD1b (n = 5) or GQ1b (n = 1), whereas positivity for IgM anti-ganglioside antibodies was found for ganglioside GM1 (n = 1), GM1b (n = 1), GD1b (n = 1) or GQ1b (n = 1). None of the *H. influenzae*-positive patients showed evidence of recent infection by *C. jejuni*, CMV, EBV or *M. pneumoniae*.

**Relationship of *H. influenzae*-positive Guillain–Barré syndrome to axonal Guillain–Barré syndrome and to anti-ganglioside antibody**

On the basis of their peripheral conduction abnormalities, 46 Guillain–Barré syndrome patients were classified as having AIDP (n = 18) or AMAN (n = 21), or were unclassified (n = 7). Of the 21 with AMAN, 19% (n = 4) were *H. influenzae*-positive.

Of the 46 patients with Guillain–Barré syndrome, 35 had IgG or IgM anti-ganglioside antibodies. IgG anti-ganglioside antibodies were found for the gangliosides GM1 (n = 20), GM1b (n = 17), GD1a (n = 14), GalNac-GD1a (n = 8), GD1b (n = 12) and GQ1b (n = 5), and IgM anti-ganglioside antibodies were found for GM1 (n = 6), GM1b (n = 3), GM2 (n = 3), GD1b (n = 1) and GQ1b (n = 1). Anti- *H. influenzae* antibody was present in 17% of the patients carrying IgG and/or IgM anti-ganglioside antibodies, 25% of those with IgG anti-GM1 antibody, 42% of those with IgG anti-GD1b antibody and 29% of those with IgG anti-GD1a antibody.

**Discussion**

The aim of this retrospective study was to clarify the association between *H. influenzae* infection and Guillain–Barré syndrome using a case–control design, and to clarify the clinical and laboratory features of Guillain–Barré syndrome subsequent to *H. influenzae* infection. We found that a form of Guillain–Barré syndrome occurs after respiratory infection by *H. influenzae* in the Japanese population. Guillain–Barré syndrome patients with *H. influenzae* infection were characterized clinically as having had a preceding respiratory tract infection, less frequent cranial and sensory nerve involvement, and good recovery. Moreover, they frequently
Table 1 Clinical features of H. influenzae-positive Guillain–Barré syndrome patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age/sex</td>
<td>23/F</td>
<td>33/F</td>
<td>41/F</td>
<td>30/M</td>
<td>25/F</td>
<td>80/F</td>
</tr>
<tr>
<td>Prodromal symptoms</td>
<td>Cough, sputum</td>
<td>Cough, sputum</td>
<td>Cough, sore throat</td>
<td>Fever, sputum, sore throat</td>
<td>Fever, sputum, sore throat, rhinorrhoea</td>
<td>Right abducens</td>
</tr>
<tr>
<td>Cranial nerve involvement</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Right abducens</td>
<td>Bulbar</td>
</tr>
<tr>
<td>Sensory loss</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>All modalities</td>
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<tr>
<td>Treatment</td>
<td>IVIg</td>
<td>PP</td>
<td>PP</td>
<td>None</td>
<td>IVIg</td>
<td>PP</td>
</tr>
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<td>Disability (Hughes grade*)</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
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<td>At peak</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>1</td>
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<tr>
<td>At 4 weeks</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>At 3 months</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Time until nadir (days)</td>
<td>4</td>
<td>5</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>

*0 = healthy; 1 = minor signs and symptoms; 2 = able to walk 5 m independently; 3 = able to walk 5 m with aid; 4 = bedbound; 5 = assisted respiration required; 6 = dead. IVIg = intravenous immunoglobulin therapy; PP = plasmapheresis.

Table 2 Laboratory features of H. influenzae-positive Guillain–Barré syndrome patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum culture</td>
<td>Not done</td>
<td>H. parainfluenzae</td>
<td>Haemophilus*</td>
<td>H. influenzae</td>
<td>Negative</td>
<td>H. influenzae</td>
</tr>
<tr>
<td>IgG/IgM/IgA</td>
<td>32 000/2000/(-)</td>
<td>32 000/2000/(-)</td>
<td>64 000/(-)/1000</td>
<td>64 000/2000/(-)</td>
<td>8000/2000/(-)</td>
<td>12 800/000/(-)/1000</td>
</tr>
<tr>
<td>Anti-H. influenzae antibody titre</td>
<td>AMAN</td>
<td>AMAN</td>
<td>Isolated F wave Absence</td>
<td>AMAN</td>
<td>Isolated F wave Absence</td>
<td>AMAN</td>
</tr>
<tr>
<td>Electrophysiological diagnosis</td>
<td>GM1 (2000)</td>
<td>GM1 (4000)</td>
<td>GD1a (500)</td>
<td>GM1 (8000)</td>
<td>GM1 (500)</td>
<td>GM1 (32 000)</td>
</tr>
<tr>
<td>IgG Anti-ganglioside antibody (titre) IgM</td>
<td>GM1b (1000)</td>
<td>GD1b (500)</td>
<td>GD1a (2000)</td>
<td>GM1b (32 000)</td>
<td>GD1b (500)</td>
<td>GD1b (512 000)</td>
</tr>
<tr>
<td>GalNAc-GD1a (4000)</td>
<td>GD1b (4000)</td>
<td>GD1b (1000)</td>
<td>GM1b (2000)</td>
<td>GD1b (64 000)</td>
<td>GD1b (8000)</td>
<td></td>
</tr>
<tr>
<td>GalNAc-GD1b (4000)</td>
<td></td>
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</table>

*Unclassified Haemophilus species.

had primary axonal involvement of the motor fibres and carried anti-ganglioside antibodies. Most of these features agree with those of the Guillain–Barré syndrome associated with C. jejuni enteritis (Rees et al., 1995a; Visser et al., 1995; Jacobs et al., 1996). Our previous finding that a strain of H. influenzae has a GM1-like structure and elicits Guillain–Barré syndrome (Mori et al., 1999) is supported by the strong association between the anti-H. influenzae positivity and the anti-GM1 positivity and the results of Western blotting reported in this study.

Haemophilus influenzae is a Gram-negative human pathogen whose outer membrane contains LPS. There are two types of strain: encapsulated and non-encapsulated, non-typable (Apicella, 1994). The latter strain was used as the antigen in the ELISA carried out in this study. Previous reports suggest a causal relationship between Guillain–Barré syndrome and the H. influenzae type b (encapsulated type) vaccine, which is composed of the capsular antigen of the bacteria (D’Cruz et al., 1989; Gervaix et al., 1993), but subsequent evaluation has not confirmed this association (Gross and Hayes, 1991). The non-typable (non-encapsulated) H. influenzae, however, has been shown to be an important pathogenetic cause of community-acquired bacterial pneumonia in adults (Apicella, 1994), but had not been described as a pathogen of Guillain–Barré syndrome until association between the anti-H. influenzae positivity and the anti-GM1 positivity and the results of Western blotting our previous report (Mori et al., 1999).

Jacobs and colleagues reported that only 1% of 154 patients with Guillain–Barré syndrome had elevated anti-H. influenzae antibody and that there was no significant difference in the presence of the antibody when compared with the controls, including patients with other neurological diseases (Jacobs et al., 1998). There are a number of reasons for the different frequencies of Guillain–Barré syndrome patients with anti-H. influenzae antibody in their study and ours. Jacobs and
The assay is more sensitive than the IgM assay for C. jejuni neuropathic symptoms. In fact, the IgG anti-IgM or IgA antibody may decrease until the onset of

H. influenzae from purulent sputum specimens of two H. influenzae patients in Western countries, where anti-GM1 antibody has a nadir of the disease and could run within 3 months. Moreover, Western blot analysis showed the presence of IgG antibodies against H. influenzae LPS in all the H. influenzae-positive patients, whereas these antibodies were not present in CMV-positive or C. jejuni-positive Guillain–Barré syndrome patients. We speculate (i) that there is an H. influenzae strain that causes Guillain–Barré syndrome, (ii) that this strain, like a certain C. jejuni strain, may carry a GM1-like epitope on its LPS, and (iii) that molecular mimicry between the ganglioside and the bacterial epitope may cause nerve damage because of an immune response. The similarities between Guillain–Barré syndrome subsequent to H. influenzae infection and C. jejuni infections may reflect the same pathophysiology. Despite the high incidence of H. influenzae infection in the overall population, Guillain–Barré syndrome is a rare disease, probably because only a particular strain causes Guillain–Barré syndrome or because persons who develop the disease differ in their responses to the infection.

In our study, all the H. influenzae-positive Guillain–Barré syndrome patients could walk unaided within 4 weeks of the nadir of the disease and could run within 3 months. Moreover, there seemed to be a better prognosis than that previously described for C. jejuni-related Guillain–Barré syndrome patients (Rees et al., 1995a; Visser et al., 1995; Jacobs et al., 1996). Some reports have denied that there is a relationship between anti-GM1 antibody and C. jejuni infection or between anti-GM1 antibody and a poor prognosis (Enders et al., 1993; Vriesendorp et al., 1993). We found no significant correlation between positivities for anti-GM1 and anti-H. influenzae antibodies. This may be because of the heterogeneity of the anti-GM1 antibody-positive patient group, which consisted of at least two subgroups of patients with Guillain–Barré syndrome subsequent to H. influenzae infection: those without anti-C. jejuni antibody who had a good recovery and those with Guillain–Barré syndrome subsequent to C. jejuni infection who had a relatively poor recovery (Kuwabara et al., 1998a).

In conclusion, H. influenzae infection elicits an axonal form of Guillain–Barré syndrome associated with anti-ganglioside antibodies similar to those present in C. jejuni-related Guillain–Barré syndrome, but there is better recovery from the H. influenzae-related form of the disease.

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