Myelin- and Microbe-Specific Antibodies in Guillain-Barré Syndrome

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We surveyed the frequency of reported infections and target autoantigens in 56 Guillain Barré syndrome (GBS) patients by detecting antibodies to myelin and microbes. Sulfatide (43\%), cardiolipin (48\%), GD_{1a} (15\%), SGPG (11\%), and GM_{3} (11\%) antibodies were the most frequently detected heterogeneous autoantibodies. A wide spectrum of antimicrobial IgG and IgM antibodies were also detected; mumps-specific IgG (66\%), adenovirus-specific IgG (52\%), varicella-zoster virus-specific IgG (48\%), and S. pneumoniae serotype 7-specific IgG (45\%) were the most prevalent. Our results indicate that polyclonal expansion of physiologic and pathologic antibodies and/or molecular mimicry likely occurs following infection and is related to other autoimmune factors in the etiology of GBS. Although no single definitive myelin-specific autoantibody was identified, our results suggest a unique pattern of reactivity against autoantigens. © 1995 Wiley-Liss, Inc.

Key words: autoantibodies, autoimmunity, gangliosides, myelin, neuropathy

INTRODUCTION

Guillain-Barré syndrome (GBS) is a transient neurological disorder characterized by areflexic motor paralysis with mild sensory disturbances in conjunction with an acellular rise of total protein in the cerebrospinal fluid associated with an inflammatory demyelination of peripheral nerves (1,2). Although the pathogenesis of GBS has not been fully elucidated, there is increasing evidence pointing to an autoimmune etiology (3,4). Autoantibodies to various myelin-associated glycoconjugates are described in GBS patients (5,6). However, the primary autoantigen in GBS is not yet clearly identified. Moreover, it is not yet fully determined whether the autoantibodies associated with GBS are directly involved in the pathogenesis of nerve damage or induce demyelination and release of autoantigens via a T-cell-mediated inflammatory reaction (7,8). Successful treatment of some GBS patients by plasmapheresis and intravenous gammaglobulin (IVIG) supports the notion that circulating myelin autoantibodies could play a direct role in pathogenesis. Myelin-specific antibodies are potentially useful for diagnosis and predicting treatment success with either plasmapheresis or IVIG (9-11).

Prior infections are often associated with GBS (12,13), although the identity of the pathogen(s) and the relationship to subsequent onset of neurological disease remain uncertain. Pathogens reportedly associated with GBS include herpes viruses (HSV) (14,15), Epstein-Barr virus (EBV) (16,17), cytomegalovirus (CMV) (18,19), human herpesvirus-6 (HHV-6) (20), varicella-zoster virus (VZV) (21,22), human T-cell lymphotrophic viruses (HTLV) (23,24), human immunodeficiency viruses (HIV) (25,26), measles (27,28), coxsackievirus (29-31), rubella (32,33), mumps (34), influenza (35,36), hepatitis viruses (37,38), Mycoplasma pneumoniae (39,40), Borrelia burgdorferi (41), respiratory syncytial virus (42), Campylobacter jejuni (43,44), echovirus (45), coronavirus (46), parainfluenza (47), streptococcus (48), enterovirus (EV-70) (49), and parvovirus (50).

In this study, we surveyed sera and CSF of GBS patients for the presence of antibodies against 18 myelin autoantigens including gangliosides, galactosyl cerebroside, sulfated glycolipids, Forssman antigen, myelin basic protein (MBP), and cardiolipin. Because sequence similarities exist between bacteria, viruses, and neuronal autoantigens (51-53), this study analyzes the association

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between the presence of myelin autoantibodies and antibodies to 18 common infectious agents known to be associated with GBS. This study also attempts to identify a possible pattern of autoantibody and pathogen-specific antibody reactivity. Additionally, we used a novel flow cytometry assay to detect neuronal antibodies.

MATERIALS AND METHODS

Serum and CSF Specimens

Sera were obtained from 56 GBS patients (National Institute of Neurological Disease and Stroke diagnostic criteria; 137–139) (29 white males and 27 white females), aged 8–47 years, at diagnosis within a median period of 1 week of onset of neurologic symptoms. Age-, gender-, and race-matched normal and disease control sera were collected from 70 healthy subjects, 10 patients with clinically definite multiple sclerosis (MS) (Posner’s criteria, 10 patients with idiopathic transverse myelitis (TM) (Jeffery’s criteria), 10 patients with chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) (Uncini’s criteria), 15 patients with polyneuropathy (PN) (Pestronk’s criteria), and 16 patients with chronic fatigue and immune dysfunction syndrome (CFIDS) (CDC criteria). CSF specimens were obtained from 12 GBS patients and 10 control individuals without evidence of neuroborreliosis.

Determination of Antibodies to Myelin Glycoconjugates

Glycolipid antigens (galactosyl cerebrosides [Gal-Cer], sulfatide [ceramide galactoside 3-sulfate], SGPG [sulfoglucuronyl paragloboside], sphenomyelin, cardioliopin, Forssman antigen, monosialogangliosides [GM1, GM2, GM3], disialogangliosides [GD1a, GD1b, GD2, GD3], tri-sialoganglioside [GT1b], and asialo-GM1) and myelin glycoprotein antigens (myelin basic protein [MBP], myelin-associated glycoprotein [MAG]) were used to determine autoantibodies in sera and CSF. SGPG, MBP, and MAG antigens were prepared by extraction and chromatography (132–134). All other myelin antigens were purchased from Matreya (Pleasant Gap, PA).

Antibodies to myelin glycoconjugates were determined by enzyme-linked immunosorbent assay (EIA); 250 ng/mL of the appropriate myelin antigen was dissolved in chloroform:methanol (1:3 v/v) and coated by evaporation in flat-bottom 96-well plates (Immulon 1). MBP and MAG antigens in 50 mM KHCO3, pH 9.6 (2.5 ng/mL) were coated for 24 h. Uncoated wells (background) were filled only with coating solution. The wells were blocked with phosphate-buffered saline-1% bovine serum albumin (PBS-1% BSA) for 30 min at RT and washed with PBS-T (50 mM sodium phosphate, 150 mM NaCl and 0.05% Tween-20, pH 7.4). A total of 100 μL of test sera and positive controls (diluted 1:300 in PBS-1% BSA) or undiluted CSF specimens were added to the wells and incubated for 1 h at RT. Following repeated washing, bound antibodies were detected by adding goat antihuman IgM and/or antihuman IgG conjugated to alkaline phosphatase (1:2,000 dilution) and paranitrophenyl phosphate. The optical density (OD) was read at 405 nm on an automatic microplate reader.

Determination of Microbial Antibodies

Antibodies specific for Campylobacter jejuni (IgG), human herpesvirus 6 (IHH-6) (IgG & IgM), influenza viruses A & B (IgG), Streptococcus pneumoniae (strains 3, 7, 9, 14), (IgG), Legionella pneumophila (IgG, IgM & IgA), varicella-zoster virus (VZV) (IgG & IgM), cytomegalovirus (CMV) (IgG & IgM), herpes simplex viruses (HSV-1/2) (IgG & IgM), human immunodeficiency viruses (HIV-1/2) (IgG), Mycoplasma pneumoniae (IgM), Epstein-Barr virus (EBV) (VCA IgM), and Japanese encephalitis virus (JEV) (IgM) were determined. Commercial kits were used to detect specific antibodies to HSV-1/2 (Pharmacia, Parsippany, NJ), M. pneumoniae (BioWhittaker, Walkansville, MD), and HIV-1/2 (AccuSpot™, Specialty Biosystems, San Diego, CA) according to the manufacturer’s instructions.

Specific antibodies to the other infectious agents were detected by the following generalized EIA procedure. Antigens (250 ng/mL) in 50 mM carbonate buffer, pH 9.6 were coated onto Immulon 1 (Dynatech, Chantilly, VA) or Polysorp (NUNC, Naperville, IL) polystyrene microtiter plates overnight at 4°C, decanted, washed with PBS-T, and blocked with PBS-1% BSA for 30 min at RT. Standards, controls, and test sera (diluted 1:300 in PBS-1% BSA) were pipetted in duplicate into wells and incubated for 2 h at RT on a shaker. Following repeated washing, bound antibodies were detected by adding goat antihuman IgM or antihuman IgG conjugated to alkaline phosphatase (1:1,000 dilution) and paranitrophenyl phosphate. The optical density (OD) was read at 405 nm on an automatic microplate reader.

Thin-layer Chromatography (TLC) With Immunofixation

Representative sera positive by EIA for myelin component reactivity were analyzed by TLC. A total of 5 μL each of GM1, GM2, GD1a, GT1b, and Gal-Cer were aliquoted from 1 mg/mL stock solutions to make glycolipid cocktail #1; 5 μL each of GM3, GD1b, GD2, asialo-GM1, sulfatide, and SGPG were aliquoted from 1 mg/mL stock solutions to make glycolipid cocktail #2; 5 μL of each cocktail was spotted in duplicate on 10 x 1 cm aluminum-backed silica gel plates (Aldrich, Milwaukee, WI), air dried, and developed with 50:40:5 (v/v) chloroform:methanol:0.2% KCl. The plates were air dried and briefly immersed in 0.05% polysorbbyl-methacrylate in n-hexane. After thorough drying, the fixed plates were blocked in PBS-1% BSA for 30 min, washed for
10 min in PBS, immersed in a 1:100 dilution of the appropriate serum specimen in PBS-1% BSA, and incubated overnight on a rocker. The plates were washed three times for 10 min with PBS and incubated with 1:2,000 goat antihuman IgG-biotin and 1:2,000 goat antihuman IgM-biotin conjugates (Chemicon, Temecula, CA) for 2 h at RT. After washing, the plates were incubated with 1:1,000 streptavidin-horseradish peroxidase for 1 h at RT, washed again, and developed with 1:50 diaminobenzidine substrate in 50 mM Tris-HCl, pH 7.4, and 0.01% H₂O₂. For TLC validation of glycolipid migration values (Rf), parallel plates with individual glycolipids per lane were colorimetrically stained with orcinol (gangliosides) or azure A (sulfoglycolipids).

Flow Cytometric Determination of Neuronal Antibodies

The human neuroblastoma cell lines SK-N-MC and SK-N-SH (American Type Culture Collection (ATCC), Rockville, MD) were used as a source of antigens. The neuronal cell lines were maintained in RPMI medium supplemented with 10% fetal calf serum in 5% CO₂/95% air. Confluent cells were harvested and washed twice with isotonic-buffered saline: EDTA solution (BSS). After resuspension, the cells were counted and the cell concentration was adjusted to an optimal concentration determined by block titration. Nonspecific binding sites were blocked by incubating the cells with BSS containing 10% goat serum. After incubation, cells were repeatedly washed and resuspended in BSS; 50 μl aliquots of the cell suspension were dispensed into incubation tubes and 50 μl of diluted sera were added to the tubes. The cells were incubated, repeatedly washed, and stained with FITC-labeled goat antihuman IgG conjugate. After further washing, the cells were resuspended in BSS and analyzed in an EPICS Profile flow cytometer. The mean intensity of fluorescence (MIF) was determined for each sample. Cutoff values were defined as the mean + 2 standard deviations of MIF units using the frequency distribution curve of healthy control sera at the 95% confidence limit (5 MIF units).

Statistical Analyses

The statistical analyses included testing for normal distribution, Student’s t-test and Spearman rank correlation. Accuracy and precision of each EIA run was estimated using 70 normals to establish the cutoff (mean ± 3 SD). Corrected values of P < 0.05 were considered significant for t-test analyses, as were Rs values ≥ 0.49 in the Spearman rank correlation tests.

RESULTS

The interassay coefficient of variation (CV) of the cutoff value was ±15% for each glycolipid antibody EIA and ±5% for each microbe-specific antibody EIA. Various autoantibodies were detected in the sera and CSF of GBS patients (Table 1); none of the antibodies alone could be considered diagnostic (<50% reactivity). Cardiolipin (48% [27/56]), sulfatide (43% [24/56]), and GD₁₀ (14% [8/56]) were the most frequently detected antibodies in GBS sera followed by GM₁, GM₁₀, and SGPG antibodies (11% [6/56]). None of the healthy controls had antibodies to these autoantigens. Neuronal antibodies were not detected in any of the GBS patient sera. Overall, 52% (29/56) of the GBS patient sera had at least one antibody against one of the myelin autoantigens; reactivity against more than five autoantigens was noted in at least six sera (up to 14 autoantigens) (Table 2). Multireactivity to 10 or more autoantigens was found in all CSF specimens from GBS patients (Table 1). Of the 12 GBS CSF specimens, 100% (12/12) reacted with GD₁₀ antigen and 92% (11/12) reacted with each of 10 other autoantigens. The specificity of CSF reactivity was confirmed with the control CSF from individuals without evidence of neuroborreliosis. None of these control specimens (0/10) were reactive with any myelin component.

Representative sera positive for autoantibodies to myelin components by EIA were confirmed by TLC with immunofixation. The sera tested were multireactive by EIA; this multireactivity was confirmed by TLC with up to 10 bands detected (r = 0.99) (Fig. 1); no bands were detected in normal control sera.

Specific IgG antibodies to S. pneumoniae (34–45%), adeno- virus (52% [29/56]), mumps (66% [37/56]), VZV antibodies (46% [26/56]), and influenza A/B antibodies (23% [13/56]) were commonly detected in GBS patient sera (Table 3). Specific IgM antibodies to Campylobacter jejuni (20% [11/56]), CMV (13% [7/56]), HHV-6 (7% [4/56]), VZV (11% [6/56]), mumps (16% [9/56]), HSV-1 (18% [10/56]), M. pneumoniae (11% [6/56]), HSV-2 (9% [5/56]), EBV- VCA (4% [2/56]), and JEV (4% [2/56]) were also detected in GBS patient sera. A multireactive pattern of both IgG and IgM microbe-specific antibodies were also observed in GBS sera; some patients had antibodies to as many as 15 infectious agents (Table 4). Only eight patients lacked antibodies to any of the above microorganisms.

Various levels of significance between the sera multireactive for autoantigens and microbes were established by Spearman rank correlation (Table 5). A significant relation of mumps IgG and sulfatide antibodies is illustrated (see Fig. 2) by linear regression analysis. One patient with 10 glycolipid autoantibodies had antibodies against C. jejuni only, whereas another patient had one autoantibody and antibodies to eight different microorganisms (Table 6). The majority of autoantibodies were of low titer polyreactivity (Fig. 2), although some higher affinity autoantibodies of perhaps increased specificity and pathogenicity were also detected (54,55).

To determine whether transient sulfatide autoantibodies (56) were present in mumps IgG-positive sera from individuals without GBS or other clinical autoimmune diseases, control mumps IgG-positive sera were tested for sulfatide
antibodies. Eighteen percent (2/11) of control mumps IgG-positive sera were positive for sulfatide antibodies (data not shown). Preincubation of up to 1 µg of sulfatide with GBS and control mumps-positive sera did not inhibit binding to mumps antigen-coated plates.

DISCUSSION

GBS is an acute condition that usually resolves within a few days to a few weeks and responds to immunomodulation with IVIG and IgG-clearing modalities such as plasmapheresis. During the last three decades, ample information indicates that GBS could have an autoimmune etiopathogenesis (57–59).

Wide discrepancies and variations are reported in both the type and binding characteristics of the autoantibodies associated with GBS and the potential infectious causes of the disease. Autoantibodies reported in GBS include mainly antibodies to gangliosides such as GM1 (60,61), GD, (62), asialo-GM1 (63), GQ1b (64,65), and 3’-LM1 (66) as well as antibodies to galactosylcerebroside (67,68) and sulfated structures such as SGPG and sulfatides (69,70), the Forssman-like antigen (71), and to myelin proteins such as P0 (72), myelin oligodendrocyte glycoprotein (MOG) (73) and P2 (74). Furthermore, specific T-cell reactions to P0 and P2 antigens were recently described (75,76). Autoantibodies to phospholipids such as cardiolipin and phosphatidylcholine (77,78), as well as β-tubulin (79), neuron-specific enolase (NSE), S-100b protein (80), antineutrophil cytoplasmic antibodies (ANCA) (81), and double-stranded DNA (82) are also described. The frequencies reported for the appearance of these various autoantibodies in GBS range from 5–50%.

TABLE 2. Polyreactivity of GBS Sera Against 18 Myelin Antigens

| # of antigens | # of sera 29/56 |
|---------------|----------------|
| 1             | 3              |
| 2             | 16             |
| 3             | 2              |
| 4             | 1              |
| 5             | 1              |
| 6             | 1              |
| 8             | 1              |
| 9             | 1              |
| 10            | 1              |
| 13            | 1              |
| 14            | 1              |

P = Patient
N = Normal

**Fig. 1.** TLC of representative glycolipid-reactive GBS serum vs. normal control.
Conserved epitopes could also be distributed in organ systems other than the nervous system (83–88,106). Current evidence supports the notion that there is no single GBS antigen. Likewise, there are multiple antecedent factors (i.e., infectious agents) for the disease, and the different myelin antigens might be related in terms of sequential and conformational homology to the various pathogens (molecular mimicry) (89–93). GBS can also occur in association with other autoimmune diseases (neurologic overlap syndrome) (130).

Although some autoantibodies (cardiolipin, GD_{1a}, sulfatide, GM_{1}, and SGPG) were detected at a higher frequency in sera of GBS patients, none of these autoantibodies can be excl-
Fig. 2. Titers of antibodies to myelin sheath components as measured by EIA: a = sulfatide, b = cardiolipin, c = GD1a, d = GM3, e = GM1.
REGRESSION OF MUMPSG ON SULF

Fig. 3. Regression analysis of mumps IgG (OD405) vs. sulfatide (OD405).
tively used as a diagnostic parameter because they were detected in <50% of the patients. Interestingly, 11% of the patient sera reacted with more than five structurally diverse autoantigens (MBP, gangliosides, and sulfatide). Patterns of glycolipid cross- and/or poly-reactivity are also noted in IgM paraproteinemia associated with polyneuropathy (94) and amyotrophic lateral sclerosis (95). Of all GBS patient sera, 52% reacted with one or more of the myelin antigens; the highest frequencies of reactivity were observed with cardiolipin (48%) and sulfatide (43%). The finding that 100% and 92% of GBS CSF specimens were reactive with GD1a and each of 10 other autoantigens, respectively, indicates that CSF might be a more appropriate specimen for evaluation of GBS and suggests possible sequestration of circulating autoantibodies in the central nervous system (96) and/or intrathecal synthesis of immunoglobulins (97,98). However, most CSF myelin antibody titers were low and the specimens were polyreactive. Elevated CSF myelin antibodies (relative to sera) were previously described in neuroborreliosis, chronic inflammatory demyelinating polyneuropathy, and GBS (131).

GBS sera also showed a wide spectrum of microbe-specific reactivity. Indeed, IgM antibodies were detected against both common as well as infrequent infections in these patients. For example, C. jejuni-specific IgM was detected in 20% of patients, whereas IgM antibodies to HSV-1/2, mumps, VZV, HHV-6, CMV, and M. pneumoniae were detected in 11–18% of patients. Furthermore, high titers of IgG antibodies were detected in some patients. The high correlation of mumps and sulfatide reactivities, as well as the specificity of mumps reactivity, suggests polyclonal activation of lymphocytes by mumps infection as a pathogenic mechanism rather than true cross-reactivity (99). Further work with immunoaffinity fractionated mumps-positive sera is in progress.

There is currently much discrepancy in the literature concerning the clinical and analytical (i.e., endpoint titration versus concentration [AU/L]) specificity and sensitivity of sulfatide antibodies. Depending on the method of detection, sulfatide antibodies are reported in 5 to >50% of GBS patients (6,100). The prevalence of sulfatide antibodies in other neurologic, immunologic, and autoimmune diseases and healthy controls emphasizes the current uncertainty of the clinical utility of sulfatide antibody detection (85,101). Definitive cohort studies with acute and convalescent specimens are needed to assess whether detectable “natural” sulfatide autoantibodies predispose patients to neuropathy and autoimmune disease and whether low titers progress to elevated and pathological levels. Antibody avidity studies (55) are also required to discriminate between “natural” and pathogenic sulfatide antibodies.

Similar problems of interlaboratory assay standardization, definition of assay cutoffs, and differences in diagnostic categorization as well as patient and control selection criteria are reported for GM1 antibodies in motor neuron disease (102). However, high titer and/or high activity sulfatide, cardiolipin, and other glycolipid antibodies are usually confirmatory for neuropathy, nervous system inflammation, and neuropsychiatric involvement. High titer sulfatide antibodies (defined to give ≥98% clinical specificity; cut-off = 2) were detected in 14% of the GBS patients in this study. The high specificity of CSF GD1a antibodies supports the diagnostic utility of GD1a antibodies in “severe GBS” (103). Further evaluation of the clinical specificity of GD1a antibodies is in progress. The reactivity of the GBS sera to the 18 myelin autoantigens evaluated suggests a unique pattern of myelin-specific antibodies.

Our results suggest a multi-infectious etiology of GBS or an increased susceptibility of GBS patients to infections and illustrate the difficulties in determining whether infection by a specific organism triggers the disease in individual patients. Interestingly, a recent report describes a patient in whom each recurrence of GBS was preceded by different infections (104). The reported predominance of upper respiratory or gastrointestinal tract infections in GBS patients was not confirmed in our studies because pathogens with various tissue specificities were detected in most of the GBS patients evaluated.

Our results indicate that autoantibody screening is potentially useful for early detection of autoimmune disease onset following certain infections, especially mumps, CMV, HSV, S. pneumoniae, VZV, adenovirus, C. jejuni, and Influenza A/B (Table 6), and that patients with certain autoimmune disorders should be tested for appropriate infectious diseases (105–108). Appropriate autoantigen and infectious agent testing is advised in insulin-dependent diabetes mellitus (mumps, CMV, coxsackieviruses [109,110]), Sydenham's chorea, Chagas disease, and myocarditis (Trypanosoma cruzi and CMV [85,111,129]), reactive and rheumatoid arthritis (Streptococcus, Yersinia, and Klebsiella [48,112,113]) and in multiple sclerosis (measles, rubella, varicella-zoster [MRZ reaction] [135]).

We believe that GBS is both a cellular and humoral autoimmune disease induced by infection with multiple microorganisms and that the presence of microbe-specific antibodies and T cells with cross-reactivity to various nerve-sheath components initiate inflammatory demyelination and shedding of peripheral nerve autoantigens (114). Infection can result in polyclonal expansion and elevation of physiological autoantibody concentration (54,115,116). In the presence of cytokine-induced antigen presenting cells (e.g., Schwann cells) (117), autoantigens are presented to the immune system leading to secondary T-cell mediated activity, which together with loss of tolerance, B-cell proliferation, and autopspecific affinity maturation could be responsible for the progressive and sometimes chronic changes and dysfunction in GBS patients (118). Autoantigens can also cause proliferation of activated T cells in a dose-dependent manner through the idiotype network (119). Tertiary suppressor T-cell responses are noted in spontaneous recovery from
anti-idiotypic antibodies (120,121), and ganglioside-induced experimental demyelination (120,121), as well as apoptosis of secondary and primary T cells (122), the induction of immunosuppression of lymphocytes (123). If instituted early, both plasmapheresis and IVIG can interfere with the progression of the disease and promote recovery, although in some instances plasmapheresis and IVIG are contraindicated (125,126,128). Corticosteroid treatment is effective in some cases (127) and can work synergistically with IVIG (128).

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