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Anti-GM_1 IgG antibodies in Guillain–Barré syndrome: fine specificity is associated with disease severity

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ABSTRACT

Background Clinical severity of Guillain–Barré syndrome (GBS) is highly variable, but the immunopathological reason is unknown.

Objective The study was designed to show which antibody parameters are associated with disease severity in GBS patients with serum anti-GM1 IgG antibodies.

Methods Thirty-four GBS patients with anti-GM_1 IgG antibodies were grouped into two categories according to disease severity at nadir: mild (grades 1–3 by Hughes functional scale, n=13) and severe (grades 4 and 5, n=21). Titre, affinity, fine specificity and cell binding of anti-GM_1 antibodies were obtained and compared between the two groups.

Results No differences in antibody titre (GM_1-ELISA) or affinity were found between the two patient groups. In contrast, the severe group showed a significantly higher frequency (95%, vs 46% in the mild group, p=0.002) of specific (not cross-reacting with GD_{1b}) anti-GM_1 antibodies. In addition, the severe group also exhibited a higher antibody binding titre to cellular GM_1.

Conclusions Differences in fine specificity of antibodies are strong indications that different regions of the GM_1-oligosaccharide are involved in antibody binding. High titres of specific anti-GM_1 antibody binding to cellular GM_1 can be explained by antigen exposure, that is, GM_1 exposes or forms mainly epitopes recognised by specific antibodies, and 'hides' those involved in binding of cross-reacting antibodies. Thus, the fine specificity of anti-GM_1 antibodies may influence disease severity by affecting antibody binding to cellular targets. Additionally, since antibody specificity studies are relatively easy to implement, fine specificity could be considered a useful predictor of disease severity.

INTRODUCTION

Guillain–Barré syndrome (GBS) is an acute, mainly motor neuropathy caused by an autoimmune process.[1] It is a self-limited disease, with muscle weakness reaching a nadir within 4 weeks, followed by partial or complete recovery. GBS is divided into two major subtypes, acute inflammatory demyelinating polyneuropathy and acute motor axonal neuropathy (AMAN), based on immunohistological studies.[2 3] Severity at nadir is highly variable, from patients with minor weakness, to patients requiring respiratory assistance, even to death.[1] The reason for this variability is unknown, although some factors predicting a poor outcome have been identified.[4] Anti-GM_1 IgG-antibodies are frequently found in AMAN patient sera,[5] and sensitisation of rabbits with GM_1 produces a replica of AMAN.[6 7] Whether anti-GM_1 antibodies are associated with a clinical severity at nadir is controversial.[5 8 9] Anti-GM_1 IgG antibodies found in GBS patients belong to the IgG1 or IgG3 subclass.[10 11] At nadir, the disease severity of patients having either subclass is similar, but the presence of IgG1 is a predictor of slow clinical recovery.[10 11] Here we describe characterisation of the anti-GM_1 IgG antibodies present in GBS patients, and a search for antibody properties that can explain the clinical variability of the disease.

MATERIALS AND METHODS

Patients

Sera from 34 GBS patients carrying anti-GM_1 IgG antibodies were collected at Dokkyo Medical University, Japan, with prior approval from the Ethics Committee. Serum samples taken during the first 3 weeks after the onset of disease (before immune treatment) were stored at −80°C until use. Prior to being transported to the Argentine laboratory by an international courier service, samples were lyophilised. Previous experiments carried out with human and rabbit sera indicated that these operations do not modify activity of anti-GM1 anti-ganglioside antibodies. All the procedures using human sera were approved by the Internal Committee of CIQUIBIC-CONICET. Criteria for inclusion were a positive spot for GM_1 or GM_1 and GD_{1b} in thin-layer chromatogram (TLC)-immunostaining at 1/200 dilution, and no spot for other gangliosides. Anti-GM_1 IgG antibody binding was characterised by blind assay, and correlation of various antibody parameters with disease severity was tested.

Patient disability was evaluated using the Hughes functional grading scale,[12] that is, grade 1=minor symptoms, able to run; grade 2=limb weakness, able to walk 5 m unaided; grade 3=able to walk 5 m only with aid; grade 4=chair or bed bound; grade 5=requiring assisted ventilation. Patients able to walk at nadir (Hughes grades 1–3) were considered having a mild disease (n=13), while a severe condition was assigned to those having grades 4–5 (non-walking patients).

Glycolipids

GM_1, GD_{1a} and GD_{1b} were obtained from human brain. Folch upper phase was purified by DEAE chromatography,[13] and HPLC on Iatrobeads silica gel column,[14] in order to obtain highly purified gangliosides.

Enzyme-linked immunosorbent assay (ELISA)

Twenty-five picomoles of GM_1 in 50 µl of methanol was pipetted into microtitre plate wells,

Table 1 Clinical features of patients with Guillain–Barré syndrome

Patient no	Age (years)	Sex	Antecedent diarrhoea	Hughes functional grade	Anti-GM1 IgG antibodies	
					Specificity	High affinity*
1	59	M	+	5	S/C	+
2	58	F	+	4	S	+
3	29	M	–	4	S	–
4	59	M	+	4	S	–
5	61	M	+	4	S	–
6	53	F	+	4	S	+
7	31	F	+	4	S	+
8	20	M	+	4	C	+
9	18	M	–	4	S	+
10	26	M	+	4	S/C	+
11	12	F	+	4	S/C	+
12	76	F	+	4	S	+
13	35	F	–	4	S/C	–
14	15	M	+	4	S/C	+
15	12	M	+	4	S/C	–
16	40	F	+	4	S	+
17	64	M	–	4	S	+
18	9	M	+	4	S/C	+
19	5	F	+	4	S	+
20	35	F	+	4	S	+
21	36	M	+	4	S	+
22	60	M	+	3	S	–
23	47	M	+	3	S	–
24	38	F	–	3	C	+
25	36	M	+	3	S	+
26	33	M	+	3	C	+
27	33	M	–	3	C	+
28	52	M	+	2	C	+
29	32	F	+	2	S/C	+
30	16	F	–	2	C	+
31	26	M	+	2	C	+
32	61	M	+	2	C	–
33	51	M	+	2	S	+
34	39	M	–	1	S	+

*Presence of antibody-binding inhibition using a [GM1]=10^{-9} M.
C, cross-reactive with GD1b; F, female; M, male; S, specific.

which were then dried at 37°C overnight, and blocked with bovine serum albumin (BSA)–phosphate-buffered saline (PBS) (1% BSA in PBS) for 1 h. Each well was added with BSA–PBS diluted serum (50 µl), incubated overnight, and washed with PBS. Binding was detected following 2 h incubation with BSA–PBS diluted (1/1000) peroxidase-conjugated goat anti-human IgG. All incubation steps were performed at 4°C. After washing, colour was developed in a substrate solution containing 15 mM o-phenylenediamine and 0.015% H_2O_2 in 0.1 M sodium acetate buffer, pH 5.0, at room temperature. The reaction was stopped after 30 min by the addition of 100 µl of 1 M H_2SO_4, and OD was measured at 450 nm. Non-specific antibody binding (OD value from a well not containing glycolipid) was subtracted from each measurement. All samples were analysed in duplicate. Titre values were calculated as the reciprocal of the serum dilution needed to obtain half-maximal antibody binding.[15]

TLC-immunostaining

TLC plates were used to separate 0.3 nM of each glycolipid in running solvent chloroform/methanol/aqueous 0.2% $CaCl_2$ (45:45:10), using a tank designed to obtain highly reproducible chromatograms.[16] After air-drying, plates were coated by dipping for 2 min in a 0.5% solution of polyisobutylmethacrylate (Sigma, St Louis, Missouri) in n-hexane/chloroform (9:1). Plates were blocked with BSA–PBSt (1% BSA in PBS containing 0.05% Tween 20) for 1 h, incubated overnight with BSA–PBSt diluted serum (1/10), and washed thoroughly with PBSt. Binding was detected following 2 h incubation with BSA–PBSt diluted (1/1000) peroxidase-conjugated anti-human IgG (γ chain) goat antibodies (Sigma). All incubation steps were performed at 4°C. After washing, colour was developed in a substrate solution containing 2.8 mM 4-chloro-1-naphthol and 0.01% H_2O_2 in methanol–20 mM Tris-HCl buffer, pH 7.4 (1:29). The reaction was stopped after 20 min by washing the plates with PBSt. For quantitative studies, spots were measured by densitometry scanning at 590 nm.

Soluble antigen-binding inhibition assay (SABIA) and affinity estimation

Fine specificity was defined by SABIA. TLC plates containing GM_1, GD_{1a} and GD_{1b} were incubated with non-treated patient sera or sera preincubated for 1 h with 10^{-5} M soluble GM_1 or GD_{1b}.[17] (The word 'soluble' used here denotes a physical state of GM_1 different from solid-phase adsorbed GM_1. In reality, glycolipid-detergent mixtures do not form real solutions, but form micellar dispersions). Antibody affinity for GM_1 was estimated by quantitative SABIA, considering antibody binding inhibition by 10^{-9} M GM_1 as an indication of the presence in serum of a high-affinity antibody population. The percentage of inhibition was considered to indicate the relative size of such population.

Cell-ELISA

Clone 4 is a CHO-K1 (ATCC) stable clone obtained by double-transfection of wild-type cells with UDP-GalNAc: LacCer/GM_3/GD_3 N-acetyl-galactosaminyltransferase (GalNAc-T, EC 2.4.1.92) and UDP-Gal:asialo-GM_2/GM_2/GD_2/galactosyl-transferase (Gal-T2, EC 2.4.1.62).[18] Wild-type CHO-K1 cells and Clone 4 cells were grown in 96-well tissue culture plates until confluence, washed with PBS and fixed with 2% p-formaldehyde for 15 min. After washing and blocking with 3% BSA in PBS, wells were added with serially diluted serum. The antibody binding protocol was as described above (ELISA section). OD values for CHO-K1 cells were subtracted from those for Clone 4 cells, for each serum dilution. Titre values were calculated as the reciprocal of the serum dilution needed to obtain half-maximal antibody binding.[15]

Statistical analysis

The tests were performed with SPSS version 11.5 (SPSS, Chicago, Illinois). For comparisons between patient groups when the data were normally distributed, the means were compared using the Student's *t* test. If the data were not normally distributed, the medians were compared using the Mann–Whitney test. Differences with p values <0.05 were considered significant. The Pearson χ^2 test was used to evaluate the frequency of high-affinity antibodies. To compare the proportions of patients presenting each of the cross-reactive antibodies or GM_1-specific antibodies, the Fisher exact test was used.

RESULTS

GBS patients were grouped into two categories based on Hughes functional grade at nadir. Distribution of age, sex and previous diarrhoea record were not significantly different in mild versus severe patients (table 1). In addition, ELISA titres

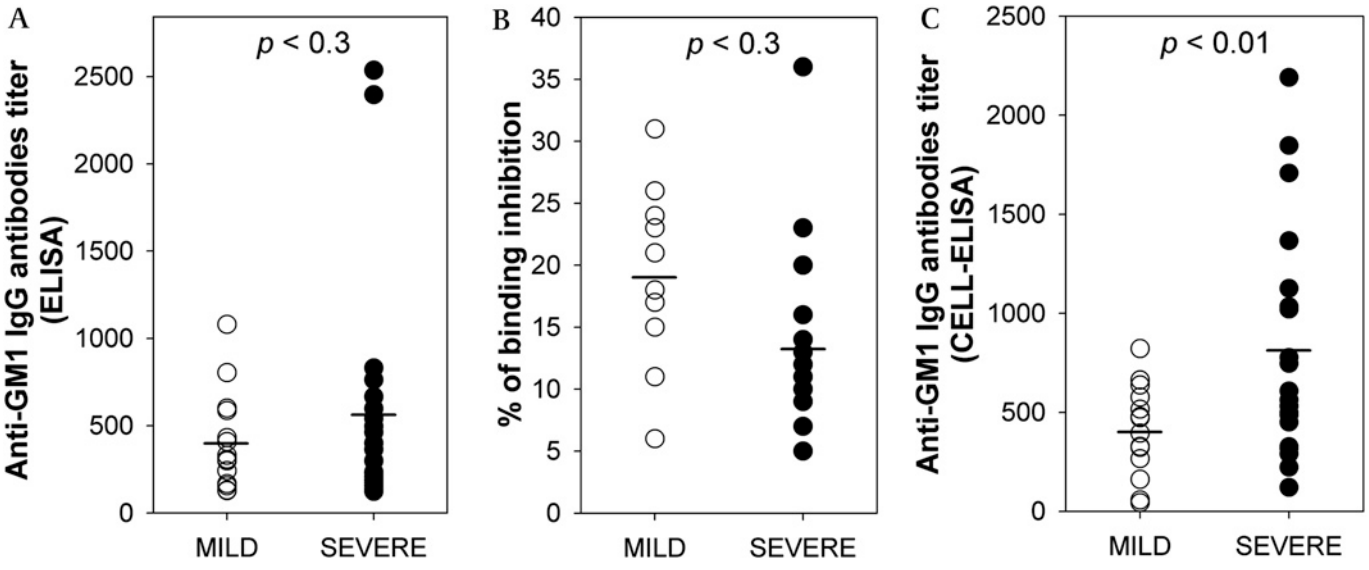

Figure 1 Anti-GM_1 IgG antibody characterisation in patients with Guillain–Barré syndrome. Sera from patients with mild (Hughes functional grades 1–3, n=13) or severe (grades 4 and 5, n=21) disease were screened by (A) ELISA, using GM_1 as antigen; (B) thin-layer chromatogram-immunostaining and soluble binding inhibition assay to detect high affinity antibodies; (C) cell-ELISA using GM_1-expressing target cells. The significance of the differences was determined by the Student's *t*.

values using GM_1 as antigen also showed no significant differences between both patient groups (figure 1A). Antibody affinity was estimated by inhibition of antibody binding by soluble GM_1.[19] Twenty-six (76%) patients had high affinity antibodies. This frequency was significantly different from that expected by random distribution (p=0.003); however, there was no difference in frequency for severe (76%) versus mild (77%) patients. The degree of the inhibition (which reflects size of the high affinity antibody population) was also similar in the two groups (figure 1B). Antibodies reactive with GM_1 may cross-react with other gangliosides. Cross-reactivity is frequently observed with GD_{1b}, a ganglioside structurally related to GM_1. Forty-seven per cent of patient sera tested gave a positive spot with this ganglioside (figure 2Ba,Ca). In all cases, antibody binding to GD_{1b} was inhibited by preincubation of sera with soluble GM_1 (figure 2Bb,Cb), indicating cross-reactivity. When the incidence of GD1b positivity was compared between both groups of patients, a difference in favour of the mild group was found (62% vs 38%). Although this difference is statistically not significant, it is not possible to discard it because of the low number of patients with anti-GD1b reactivity (table 2). Some of the sera with cross-reactive anti-GM_1 antibodies had an additional population of specific anti-GM_1 antibodies which were not inhibited by preincubation with GD_{1b} (figure 2Cc). The incidence of specific antibodies was significantly higher in the severe group (p=0.002) (table 2), indicating a close relation between the two parameters.

Preliminary results (not shown) showed binding of anti-GM_1 antibodies to cultured neuroblastoma Neuro2A cells, which express GM_1.[20] We noted a higher degree of staining with sera from severe patients, although mild patients' sera can stain these cells as well. For quantitative study of such binding, we developed a highly specific cell-ELISA assay. GalNAcT/GalT double-transfected CHO K1 cells (which express GM_1 but not GD_{1b})[18] were used as target cells. Wild-type CHO K1 cells (GM_1 negative) were used as a control, to discount non-specific

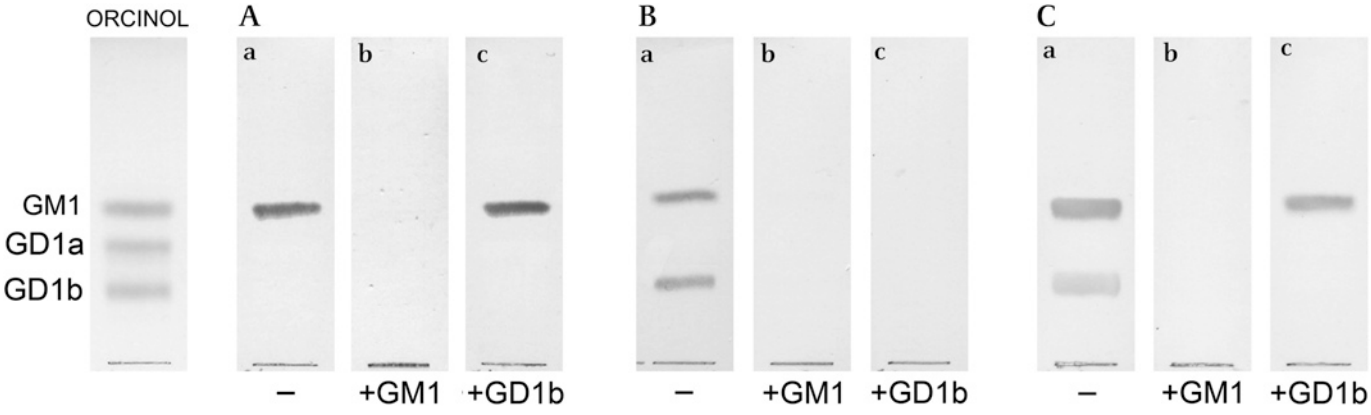

Figure 2 Fine specificity of anti-GM_1 IgG antibodies in Guillain–Barré syndrome patients. Shown are three representative patient sera with anti-GM_1 IgG antibodies which are (A) specific (not cross-reactive with GD_{1b}), (B) cross-reactive with GD_{1b}, or (C) both types. Sera were screened by thin-layer chromatogram-immunostaining as described in 'Materials and methods.' Thin-layer chromatogram plates containing GM_1, GD_{1a} or GD_{1b} were incubated with non-treated patient sera (a), or sera preincubated for 1 h with 10^{-5} M soluble GM_1 (b) or GD_{1b} (c).

Table 2 Incidence of cross-reactive and specific anti-GM_1 IgG antibodies in Guillain–Barré syndrome with mild or severe condition

Anti-GM_1 IgG antibodies*	Mild patients† (n=13)	Severe patients† (n=21)	p Value‡
Cross-reactive, n (%)	8 (62%)	8 (38%)	0.291
Specific, n (%)	6 (46%)	20 (95%)	0.002

*Antibody specificity determined by soluble antigen-binding inhibition assay.
†Patient severity assessed according to Hughes functional grading scale (mild for grade 3 or lower, severe for grade 4 or higher).
‡Fisher exact test.

binding. This approach showed a significant difference ($p<0.01$) in anti-GM_1 antibody binding between the two types of sera (figure 1C).

DISCUSSION

The severity in anti-GM_1 IgG antibody-positive GBS patients was variable. Antibody titres in severe patients were not significantly different from those in mild patients. The high affinity of anti-GM_1 antibodies has been associated with disease in rabbits[17] and humans,[19 21] but this antibody parameter does not seem to correlate with disease severity, that is, incidence of high-affinity antibody population. Moreover, its relative size is similar in the mild and severe groups. On the other hand, we found two types of anti-GM_1 antibodies in sera: cross-reactive, or not, with GD_{1b}. Apparently, cross-reactive populations were equally present in the two groups, but non-cross-reactive (specific) antibodies were found predominantly in severe patients.

Given that antibody titre and affinity are similar in the two patient groups, can the fine specificity of anti-GM_1 antibodies account for the difference in disease severity, that is, the difference in antibody binding to cell targets? Preliminary experiments suggested that, compared with mild patients' sera, severe sera give stronger immunofluorescence staining of neuron-like cells. This result is complicated by the facts that immunofluorescence of cultured cells is mainly a qualitative method, and some normal human sera mildly stain this type of cells, indicating the presence of non-specific (or not GM_1-related) antibodies. To obtain more specific and quantitative results, we developed a cell-ELISA assay in which non-specific cell binding can be evaluated for serum from each patient. Wild-type CHO-K1 cells lack complex gangliosides and contain only the simple ganglioside GM_3. These cells do not express glycosyltransferases involved in extension of the oligosaccharide chain of GM_3, but they can be transfected with genes coding for the deficient enzymes. In this way, wild-type cells were genetically modified to express GM_1 but not GD_{1b} (Clone 4).[18] The wild-type cells provide a negative control for the transfectant and can be used to discount antibody binding not related to GM_1. Because of the absence of GD_{1b} in these cells, cell binding can be attributed exclusively to GM_1, independently of the fine specificity of anti-GM_1 antibodies tested. Using this cell-binding assay, we observed a higher titre of anti-GM_1 antibodies in sera of the severe patient group.

Statistically significant associations were found between disease severity and two parameters of anti-GM_1 antibodies: cell binding and fine specificity. An important question is whether these two parameters are related, that is, can differences in cell binding be explained by the different fine specificity of anti-GM_1 antibodies? Fine specificity is a function of the different parts of the GM_1-oligosaccharide moiety involved in the binding.[22] The structure of GM_1 is included in the structure of GD_{1b} (figure 3A). GD_{1b} can be viewed as a GM_1 molecule with a certain part of the oligosaccharide moiety covered by the second NeuNAc (2-8). Anti-GM_1 antibodies that cross-react with GD_{1b} must bind to an area of GM_1 that is not covered by the second NeuNAc (figure 3B). In contrast, GM_1-specific antibodies must bind to (at least) this area. The difference in observed antibody binding to GM_1, depending on whether the antigen is studied in a cellular context (cell-ELISA) or adsorbed on a plastic surface (classical ELISA), can be explained if GM_1 is not similarly exposed in these two situations. In cells, GM_1 can interact with other membrane components in such a way that only certain areas of the oligosaccharide are exposed to the extracellular environment and capable of interacting with antibodies. Depending on which area of GM_1 is exposed, fine specificity will determine whether a particular antibody binds, or not, to the cell. If only the area covered by the second NeuNAc (2–8) of GM_1 is exposed, only specific antibodies will bind. The fact that classical ELISA does not show any differences in titres can be explained if GM_1 adsorbed on plastic surface freely exposes its oligosaccharide, allowing interaction with both specific and cross-reactive antibodies. An alternative explanation is that specific antibodies have a higher interaction with GM1 when the ganglioside is forming a complex with other membrane components.

In conclusion, our results suggest that the fine specificity of anti-GM_1 antibodies in GBS is a factor defining cell binding and consequent disease severity. This idea is consistent with the generally accepted view that glycolipids are not distributed at random in cell membranes. Rather, they are associated with cholesterol and proteins, forming heterogeneous supramolecular structures termed 'lipid rafts.'[25 26] More than half the cellular content of GM_1, in particular, is associated with membrane domains resistant to solubilisation with detergent.[27] Recently, it has been described that in cell membranes, GM_1 can be interacting with other gangliosides (mainly GD_{1a}) in a way that can regulate binding and biological activity of certain anti-GM_1 antibodies.[28]

Finally, the two antibody parameters, cell binding and fine specificity, are both potentially useful as predictor of disease severity in anti-GM_1 antibody positive patients. The cell-binding assay is complex and not suitable as a standard method in regular laboratories, but fine specificity studies are relatively simple and easily implemented.

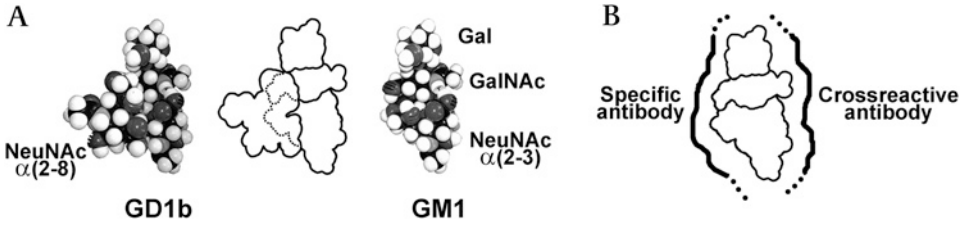

Figure 3 Schematic representation of binding of different antibody populations to GM_1 oligosaccharide. (A) Axial view ('end side' face) of a Corey–Pauling–Kortum model of GM_1 and GD_{1b} oligosaccharides, and its schematic representation. (B) Proposed areas involved in binding of different anti-GM_1 antibody populations. The Corey-Pauling-Koltun model was constructed using torsion angles as described by Acquotti and colleagues.[23 24]

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Competing interests None.

Ethics approval Ethics approval was provided by the Ethics Committee from Dokkyo Medical University, Tochigi, Japan. Internal commitee from CIQUIBIC—CONICET, Argentina.

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