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Biochimica et Biophysica Acta 1762 (2006) 357 – 361

BIOCHIMICA ET BIOPHYSICA ACTA

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A new type of anti-ganglioside antibodies present in neurological patients

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Received 6 September 2005; received in revised form 10 November 2005; accepted 14 November 2005
Available online 13 December 2005

Abstract

High titers of anti-GA_1 antibodies have been associated with neurological syndromes. In most cases, these antibodies cross-react with the structurally related glycolipids GM_1 and GD_{1b}, although specific anti-GA_1 antibodies have also been reported. The role of specific anti-GA_1 antibodies is uncertain since the presence of GA_1 in the human nervous system has not been clarified. A rabbit was immunized with GD_{1a} and its sera were screened for antibody reactivity by standard immunoassay methods (HPTLC-immunostaining and ELISA). Anti-GD_{1a} antibodies were not detected but, unexpectedly, anti-GA_1 IgG-antibodies were found. Antibody binding to GA_1 was inhibited by soluble GA_1 but also by GD_{1a}. These results indicate that the rabbit produced antibodies that recognize epitopes present on the glycolipids, that are absent or not exposed on solid phase adsorbed GD_{1a}. We investigated the presence of these unusual anti-ganglioside antibodies in normal and neurological patient sera. Approximately, 10% of normal human sera contained low titer of specific anti-GA_1 IgG-antibodies but none of them recognized soluble GD_{1a}. High titers of IgG-antibodies reacting only with GA_1 were detected in 12 patient sera out of 325 analyzed. Of these, 6 sera showed binding that was inhibited by soluble GD_{1a} and four of them also by GM_1. This new type of anti-ganglioside antibodies should be considered important elements for understanding of the pathogenesis of these diseases as well as their diagnosis.
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Keywords: Ganglioside; Anti-GD_{1a} antibody; Anti-GM_1 antibody; Anti-GA_1 antibody; Neuropathy; Motor syndrome

1. Introduction

High titers of anti-GA_1 (asialo-GM_1 ganglioside) antibodies have been associated with a variety of motor syndromes such as Guillain–Barré, Multifocal Motor Neuropathy, and Motor Neuron Disease [1–6]. In most cases they appear to be cross-reacting antibodies that recognize the Galβ(1–3)GalNAc epitope shared by GA_1, GM_1 and GD_{1b} as well as neural glycoproteins [5]. However, high titers of specific anti-GA_1 antibodies not cross-reacting with GM_1 and GD_{1b} have also been reported [4,7,8]. The exact role of these GA_1 specific antibodies is uncertain because the presence of GA_1 glycolipid in the human nervous system has not been definitively proved. Although several authors reported traces of GA_1 glycolipid in nervous tissues [9–11], there have been no experiments ruling out hydrolysis of GM_1 as a source of GA_1 during the post-mortem or the purification procedure.

In an attempt to reproduce the experimental model of neuropathy described by Nagai et al. [12], we immunized a rabbit with GD_{1a} ganglioside. The animal developed anti-GD_{1a} IgG antibodies with unusual reactivity: they bound soluble GD_{1a} but did not bind the glycolipid when adsorbed on solid phase, a condition of standard immunoassays (HPTLC-immunostaining and ELISA). Interestingly, these antibodies react with soluble and adsorbed GA_1. This result indicates the existence of anti-ganglioside antibodies with structural requirements different from those previously described. We studied the presence of these unusual antibodies in sera of patients with neurological disorders.

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doi:10.1016/j.bbadis.2005.11.005

2. Materials and methods

2.1. Human sera

Blood samples were obtained from 325 patients with neurological disorders admitted to the Neurology Division, Ramos Mejía Hospital, Buenos Aires, Argentina. They included patients with acute and chronic neuropathies with sensory and/or motor symptoms, amyotrophic lateral sclerosis, and lower motor neuron disease. Normal human blood was from healthy adult volunteers with negative serology for common infectious diseases. After clot separation (usually less than 3 h after extraction), sera were frozen at −70 °C until use.

2.2. Glycolipids

Asialo-GM_1 (GA_1) was prepared by acid hydrolysis of cow brain gangliosides [13]. GM_1, GD_{1a}, and GD_{1b} were prepared from human brain. Folch upper phase of lipid extract [14] was purified by DEAE-chromatography [15] and HPLC on Iatrobeads silica-gel column [16]. Special care was taken to obtain chromatographically pure compounds, completely freed from each other.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Twenty-five pmol glycolipids in 50 μl methanol were pipetted into microtiter plate wells. After drying at 37 °C overnight, wells were blocked with BSA-PBS (1% bovine serum albumin in phosphate buffered saline) for 1 h. Each well was added with BSA-PBSt (BSA-PBS with 0.05% Tween 20) diluted serum (50 μl), incubated overnight, and washed with PBS. Binding was detected following a 2 h incubation period with BSA-PBSt diluted (1/3000) peroxidase-conjugated goat anti-human IgG (γ chain specific; Sigma, St. Louis, MO, U.S.A.). All the incubation steps were performed at 4 °C. After washing, color was developed in a substrate solution containing 15 mM o-phenylene-diamine 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 addition of 100 μl 0.5 N 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 analyzed in duplicate. Titer values were expressed as the maximal dilution able to produce an OD value higher than 0.1.

2.4. HPTLC-immunostaining

The glycolipid mixture (0.3 nmol each of GA_1, GM_1, GD_{1a}, and GD_{1b}) was separated on HPTLC plates in the running solvent chloroform–methanol–aqueous 0.2% $CaCl_2$ (45:45:10), using a tank designed to obtain highly reproducible chromatograms [17]. After air-drying, the plates were coated by dipping for 2 min in a 0.5% solution of polyisobutylmethacrylate (Plexigum P 28, Röhm and Haas, Darmstadt, Germany) in n-hexane–chloroform (9:1). Plates were blocked with BSA-PBSt (1% bovine serum albumin in phosphate buffered saline containing 0.05% Tween 20) for 1 h, incubated overnight with BSA-PBSt diluted serum, and washed thoroughly with PBSt. Binding was detected following 2 h incubation with BSA-PBSt diluted (1/1000) peroxidase-conjugated anti-human or anti-rabbit IgG goat antibodies (Sigma, St. Louis, MO, U.S.A.). All the incubation steps were performed at 4 °C. After washing, color development was achieved in a substrate solution containing 2.8 mM 4-chloro-1-naphtol 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 water. For quantitative studies, spots were measured by densitometry scanning at 590 λ.

2.5. Soluble antigen binding inhibition assay (SABIA) and affinity studies

We tested inhibition of antibody binding to solid-phase GA_1 by soluble GA_1, GD_{1a} and GM_1. Note: the word "soluble" is used in this paper to denote a physical state of the glycolipid distinct from solid phase adsorbed one; glycolipid–detergent mixtures actually form micellar dispersion and not real solutions. Appropriated dilutions of rabbit or human sera were preincubated with antigen at various concentrations (from 10^{-11} to 10^{-4} M). After 1 h, the aliquots were assayed for antibody binding by HPTLC immunostaining. Antibody affinity for soluble GA_1, GD_{1a} or GM_1 was estimated as the glycolipid concentration that produced 50% of the maximal inhibition of antibody binding to GA_1 (IC_{50}). IC_{50} values were calculated from the slope of the straight line in a plot of 1/inhibition vs. 1/concentration.

3. Results

A rabbit was immunized with GD_{1a} in CFA and its serum screened for the presence of IgG-antibody binding to GD_{1a} and related glycolipids. Although HPTLC-immunostaining analysis showed no reactivity with the immunizing ganglioside (Fig. 1B), a remarkable result was obtained: the rabbit developed antibodies that bind to GA_1 (GD_{1a} lacking both sialic acids). Positive staining of GD_{1a} was obtained when the HPTLC plate was incubated with serum from a motor neuropathy patient containing anti-GD_{1a} antibodies (Fig. 1C), indicating that in this condition GD_{1a} can be recognized by antibodies. These antibodies are not present in preimmune sera or in rabbits injected with CFA alone (results not shown, Lopez et al. [18]). The antibodies were further characterized by soluble antigen binding inhibition assay (SABIA). Rabbit serum was preincubated with GA_1 or GD_{1a} prior to HPTLC-immunostaining assay. Soluble GA_1 completely inhibited the anti-GA_1 reactivity (Fig. 1B) but, unexpectedly, the antibody binding was also inhibited by GD_{1a}. The glycolipids used in the assays were prepared in our laboratory and are completely free of GA_1 (Fig. 1A) ruling out the possibility that inhibition is produced by a GA_1 contamination of the ganglioside preparations.

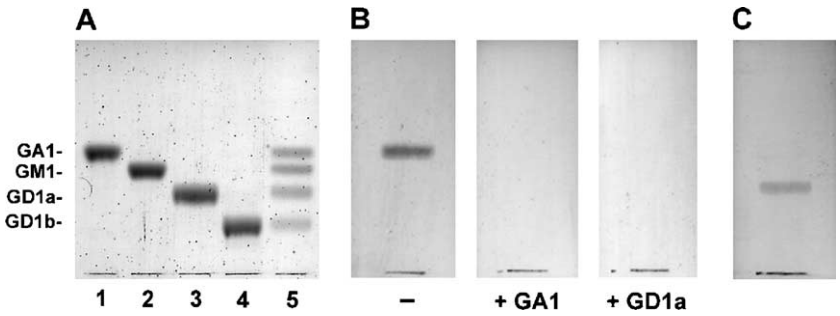

Fig. 1. Characterization of anti-ganglioside IgG-antibodies from a rabbit immunized with GD_{1a}. (A) Orcinol staining of glycolipids used in this studied: two nmol of GA_1, GM_1, GD_{1a} and GD_{1b} (1 to 4) and a mixture of 0.3 nmol of each glycolipid (5) were chromatographed on HPTLC plates. (B) Plates containing a mixture of glycolipids (0.3 nmol each) were incubated with diluted rabbit immune serum (1/50) that was previously incubated with PBS or with GA_1 or GD_{1a} (0.1 mM). Antibody binding was assayed as described in Materials and methods. (C) Immunostaining of GD_{1a} by using a patient serum containing anti-GD_{1a} IgG antibodies.

These results indicate that the rabbit produced a new type of anti-ganglioside antibodies with unusual requirement for binding to GD_{1a}. These antibodies are not detected by standard immunoassays when GD_{1a} is used as antigen, but can be measured by binding inhibition to GA_1. We searched for this type of antibodies in patients with neurological disorders and normal controls.

As we reported previously [19], plasma of ~10% of normal humans contains weak anti-GA_1 IgG–antibody reactivity. In HPTLC-immunostaining, this binding is detected at low dilution (usually 1/5) and is not inhibited by preincubation with GD_{1a} (Fig. 2). 325 neurological patients and 22 disease controls were screened for high anti-GA_1 IgG reactivity (detected at 1/20 dilution or higher) and no reactivity with GD_{1a}, GM_1, GM_3, GM_2, GD_3, GD_{1b}, GT_{1b}, GQ_{1b} and LM_1. Twelve neurological patients met this criteria and of them, 6 showed partial or total binding inhibition by soluble GD_{1a} (Fig. 2, Table 1). Inhibition with GM_1 was also observed in 4 of these 6 patients (Table 1) with not additive effect, indicating that both glycolipids are recognized by the same antibodies. ELISA determination of IgG antibody from positive patient sera showed reactivity against GA_1 (titer values founded were: 1/40; 1/160; 1/40; 1/20; 1/160 and 1/80 for patient 202; 209; 261; 262; 420 and 512 respectively) but not to GD_{1a}.

The affinity of the antibodies for GA_1, GD_{1a} and GM_1 was estimated by quantitative SABIA, considering the concentration of soluble antigen necessary to produce 50% of maximal inhibition (IC_{50}) as a relative value of antibody affinity. IC_{50} values measured for patient and control sera are summarized in Table 1. In terms of affinity for GD_{1a}, patients can be classified in two groups. The first group (low affinity) included patients with IC_{50} values equal or higher than 10^{-7} M, while in the second group (high affinity antibodies) the values ranged from 10^{-8} to 10^{-10} M. In the first group the IC_{50} values for GA_1 are similar or lower than those for GD_{1a}, whereas they are higher in the second group.

Clinical features of each patient are shown in Table 1. These unusual anti-ganglioside antibodies are most abundant among patients characterized by motor deficits and exclusively if only high affinity antibodies are considered. This observation is consistent with previous associations described for "classical" anti-GD_{1a} or anti- GM_1 antibodies [1,6,20–22].

4. Discussion

The standard methods to study anti-glycolipid antibody binding (HPTLC-immunostaining and ELISA) involve fixing the glycolipid on solid phase surface through its hydrophobic moiety. The oligosaccharide moiety is thus exposed to the medium, simulating the natural condition on the cell membrane. Antibodies reacting with glycolipids in standard methods usually also give positive reaction in cell or tissue staining, liposomes, and affinity columns. In this study, we demonstrated the presence of a new type of anti-ganglioside antibodies with behavior different from that of the "classical" antibodies—they bind soluble GD_{1a} and in some instances also GM_1, but not GD_{1a} nor GM_1 adsorbed on solid phase. Using standard methods we were able to detect these antibodies because they react with GA_1, the asialo form of complex gangliosides. The presence of anti-ganglioside antibodies with unusual binding behavior is not a surprising fact because they have been described in neurological patients [23–25].

We first detected these antibodies in a rabbit immunized with GD_{1a}, but later found them also in 6 out of 325 neurological patients. They were found mainly in patients with motor deficits, in agreement with previous reports of "classical" anti-GD_{1a} or anti-GM_1 antibodies in Guillain–Barré syndrome, multifocal motor neuropathy, and lower motor neuron disease [1,6,20–22].

A question emerges from our results: why do these antibodies recognize GA_1 and not GD_{1a} or GM_1 in standard immunoassays, while they recognize soluble form of these glycolipids? The answer to this question appears easy to explain but difficult to demonstrate: the antibodies recognize epitopes present on both molecules that are not exposed on fixed GD_{1a} or GM_1 but can be accessed when both glycolipids have less movement restriction (soluble form).

Analysis of the tridimensional structures of these glycolipids supports the role of epitope exposure on antibody binding. Spatial disposition of monosaccharides in the GA_1, GM_1, and

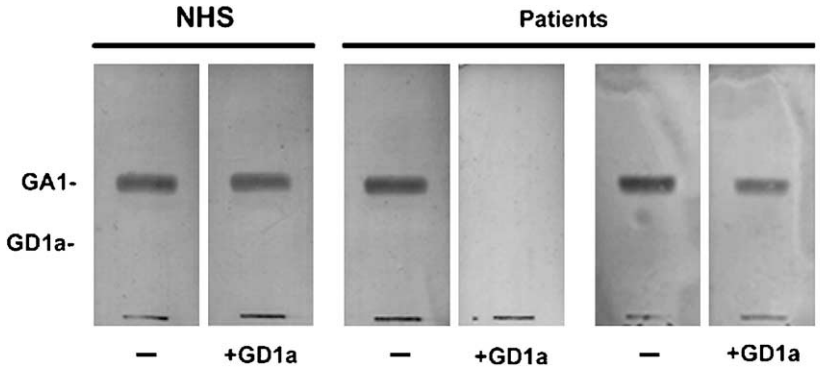

Fig. 2. Characterization of anti-GA_1 IgG antibodies in patients and normal human sera. Sera dilutions of a normal (1/5) and patients (1/20) were preincubated with 0.1 mM GD_{1a}. Two representative patient sera having anti-GA_1 antibodies with complete or partial binding inhibition by soluble GD_{1a} are shown. NHS: normal human serum.

Table 1
Estimation of antibody affinity for soluble GA_1 and GD_{1a} glycolipids

Patient	Diagnosis	Maximal inhibition by GM_1/GD_{1a} (%)	IC_{50}		
			GA_1	GD_{1a}	GM_1
NHS	–	–	2.0×10^{-5} M	NR	NR
202	MF	47	4.2×10^{-8} M	1.6×10^{-6} M	$>1.0\times10^{-5}$ M
209	AN	88	3.9×10^{-6} M	1.6×10^{-6} M	NR
261	LMND	35	ND	$>1.0\times10^{-5}$ M	NR
262	ALS	66	ND	1.6×10^{-8} M	8.8×10^{-8} M
420	MN	100	4.5×10^{-8} M	3.5×10^{-10} M	4.7×10^{-10} M
512	MN	34	3.2×10^{-6} M	2.6×10^{-10} M	$>1.0\times10^{-5}$ M

Antibody affinity was estimated by quantitative SABIA, considering the concentration of soluble antigen necessary to produce 50% of maximal inhibition (IC_{50}) as a relative value of antibody affinity. IC_{50} values were calculated from the slope of the straight line in a plot of 1/inhibition vs. 1/concentration. Sera having IC_{50} values lower than 10^{-8} M are considered that contain high affinity antibodies. NR, not reactive (not inhibited by 10^{-4} M GD_{1a} or GM_1); ND, not determined; NHS, normal human serum; MN, motor neuropathy; LMND, lower motor neuron disease; AN, ataxic neuropathy; MF, Miller Fisher syndrome.

GD_{1a} molecules has been well established by studies using NMR and minimum energy calculation [26–29]. Fig. 3 shows pictures of CPK models of GA_1, GM_1 and GD_{1a} oligosaccharide, made using the torsion angles described by Sabesan et al. [27]. The four sugars forming GA_1 and the neutral backbone of GD_{1a} and GM_1 adopt a belt shape with two clear faces: a hydrophobic face characterized by high density of C–H groups, and a hydrophilic face with a high density of hydroxyl groups. In GD_{1a} and GM_1 the NeuNAc residues are attached to the Gal residues in such a way that they cover part of the hydrophobic face. Assuming that antibodies reacting with GA_1, GM_1 and GD_{1a} should bind to shared epitopes, the epitopes should be on the hydrophilic face, because binding to those present on the hydrophobic face is sterically impeded by the NeuNAc residues in the GD_{1a} molecule. It is widely accepted that when glycolipids are fixed through the ceramide and viewed laterally, the oligosaccharide molecules resembles flower corollas attach to a ceramide stalk (Fig. 3). The Glc–Gal bond is flexible [26], allowing some freedom of movement of the oligosaccharide chain. On the other hand, the presence of hydrogen bond between the GalNAc acetamide NH and the carboxyl group of the first NeuNAc produce a structural stabilization of the oligosaccharide with restriction in the movement [26]. The first NeuNAc, GalNAc, Gal and the second NeuNAc form a relative flat "end face" that is exposed and accessible to antibodies. Classical anti-GD_{1a} antibodies would recognize epitopes on this "end face", as it was proposed for anti-GM_1 antibodies [30]. In contrast, the antibodies described here would recognize epitopes on the opposite face of GD_{1a}/GM_1 (the hydrophilic face) that is hidden or not exposed. Because of the higher mobility of the GA_1 oligosaccharide, the hydrophilic face would be more easily exposed and consequently accessible to antibodies. When GD_{1a} and GM_1 are in solution, they do not have movement restrictions and in this ways the hydrophilic face should be exposed in both molecules.

As shown in Table 1, different patients have different populations of antibodies defined by affinity and cross-reactivity. The different reactivity of the populations can be explained on the basis of their binding to different areas on the hydrophilic face. Similar affinity for two glycolipids indicates a similar area present on both molecules. Differential reactivity for GD_{1a}/GM_1 should involve the NeuNAc residues, with higher or lower affinity depending on whether they are included in the binding site or if they produce steric hindrance (Fig. 3).

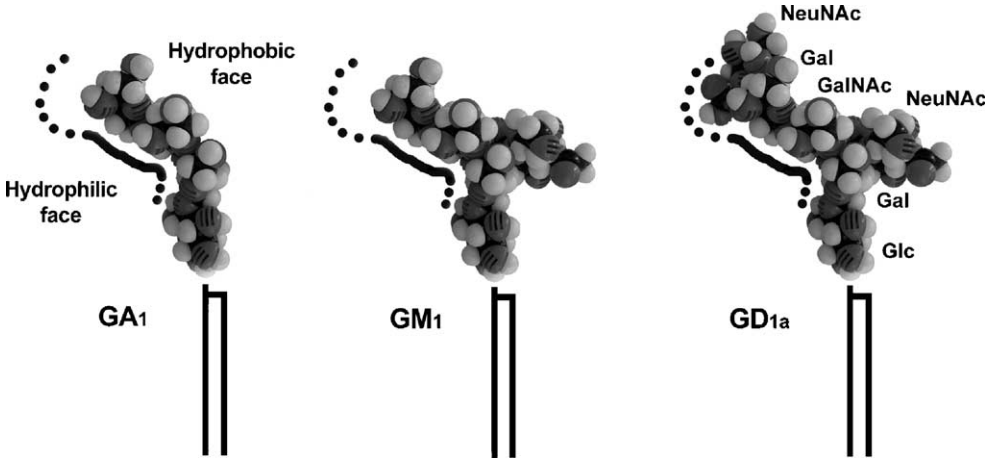

Fig. 3. Lateral view of CPK models of GA_1, GM_1 and GD_{1a} oligosaccharides, and the proposed area involved in binding of antibodies. Models were constructed using the torsion angles described by Sabesan et al. [27].

Acknowledgements

This work was supported by grants (to G.A.N.) from ISN (CAEN Grant), SeCyT (UNC), ANPCYT (PICT 05-13523) and CONICET, Argentina. P.H.H.L. and R.C. had fellowship assistance from CONICET. The authors thank Dr. F. Irazoqui for critical reading of the manuscript and Dr. S. Anderson for editing.

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