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Novel antibodies reacting with two neighboring gangliosides are induced in rabbits immunized with bovine brain gangliosides

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Immunization of rabbits with bovine brain gangliosides induced an experimental neuropathy, with clinical signs resembling Guillain–Barré syndrome. All the immunized animals developed immunoglobulin G immunoreactivity to GM1 ganglioside. In a few (4 of 27) animals, an additional anti-ganglioside antibody population showing an unusual binding behavior was detected. Enzyme-linked immunosorbent assay and thin-layer chromatography immunostaining analyses showed that the binding of these unusual antibodies required the presence of two co-localized gangliosides. Maximal interaction was observed to a mixture of GM1 and GD1b, but the antibodies also showed "density-dependent" binding to GD1b. The antibodies were purified by affinity chromatography and displayed the ability to target antigens in biological membranes (rat synaptosomes).

Keywords: density-dependent binding / experimental neuropathy / gangliosides / ganglioside complex

Introduction

During the past few decades, a large body of evidence has accumulated indicating the involvement of anti-glycan antibodies in immune responses to tumors (Hakomori and Zhang 1997). Anti-glycan antibodies have also been implicated in autoimmune processes (Gleeson 1994; Ariga et al. 2001). The epitopes of anti-glycan antibodies include two or three sugars, and the non-glycan moiety of the glycoconjugate is usually not involved in the binding. Studies so far that have characterized antibody–glycan complexes indicate the presence of a single oligosaccharide chain in the binding site (Cygler et al. 1991; Vyas et al. 2002; Krengel et al. 2004; Murase et al. 2009). However, there are other possibilities. The concept of "density-dependent binding" was proposed in the mid-1980s by Hakomori to explain the binding behavior of a melanoma-specific monoclonal antibody (mAb) that recognized the ubiquitous ganglioside GM3 (Nores et al. 1987). According to this concept, binding of the antibody requires two or more glycans fixed on a solid phase. Other examples of antibodies showing such behavior are the anti-Tn mAbs 83D4 and MLS128, whose binding requires the presence of two or three consecutive Tn antigens (Osinaga et al. 2000), and the polyclonal anti-GD1a immunoglobulin G (IgG) antibodies found in a patient with neuropathy (Kremer et al. 1997).

Following the pioneering work of Kusunoki's group (Kaida et al. 2004), several laboratories have described the presence in neuropathy patients of antibodies that recognize a complex of two different gangliosides (for review, see Kusunoki and Kaida 2011). Although the nature of the recognized epitope structure is not yet defined, a recent paper showed that an oligosaccharide dimer is involved (Mauri et al. 2012).

During the characterization of antibodies present in rabbits that develop an experimental neuropathy (resembling Guillain–Barré syndrome) following immunization with bovine brain gangliosides (BBGs), we detected some whose behavior resembled that of antibodies recognizing ganglioside complexes. Results presented here indicate that these antibodies display density-dependent binding activity.

Results

Following immunization with BBG, rabbits develop an experimental neuropathy associated with the induction of anti-ganglioside antibodies (Yuki et al. 2001; Moyano et al. 2008). The induced antibodies recognize GM1 and GD1b, two components of BBG, and GA1, the asialo form of GM1 (Comín et al. 2006; Moyano et al. 2008; Figure 1A). All of the detected immunoreactivity is related to GM1 and can be completely adsorbed using a GM1-affinity column (Moyano et al. 2008; Figure 1B). During further studies of antibodies in rabbits with neuropathy, we recently discovered a serum (termed Nx7) that displays an unusual immunoreactivity. In addition to known antibodies reacting with GM1 and GD1b, Nx7 contained IgG antibodies reacting only with GD1b,

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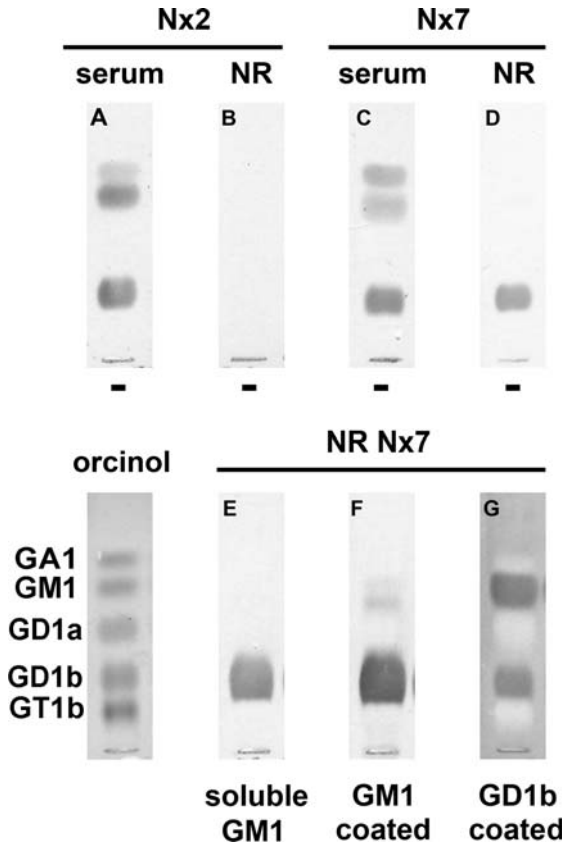

Fig. 1. Characterization of anti-ganglioside IgG antibodies present in rabbits immunized with BBG. Total serum and serum fraction NR by GM1-affinity column were analyzed by TLC immunostaining using a mixture of gangliosides as antigens. Data are shown for sera from a typical rabbit having classical anti-GM1 antibody reactivity (Nx2) and from a rabbit that also displayed an unusual immunoreactivity (Nx7). Sera or NR fractions were used without treatment (A–D, F and G) or preincubated with soluble GM1 (E). Prior to exposure to antibodies, some plates were coated with GM1 (F) or GD1b (G) by 1 h incubation with the ganglioside dissolved in PBSt. One plate was stained with orcinol reagent for chemical visualization of gangliosides.

which were found in the serum fraction not retained (NR) by a GM1 column (Figure 1D). In contrast with typical anti-GM1 antibodies, these IgG antibodies do not have IgM counterparts (results not shown). GD1b immunostaining by these antibodies showed an unusual behavior: when the NR fraction was pre-incubated with soluble GM1, a standard method for studying antibody cross-reactivity, GD1b staining was notably intensified (Figure 1E). Higher reactivity with GD1b was also observed when the TLC plate alone was incubated with soluble GM1 (Figure 1F). Following such incubation, GM1 was adsorbed over the entire TLC plate (results not shown). The reciprocal experiment gave corresponding results; that is, after the incubation of TLC plates with GD1b, we detected reactivity on the GM1 spot accompanied by a slight increase in the background staining (Figure 1G). These results can be explained if we assume that the rabbit serum contains antibodies that recognize an antigenic determinant involving both GM1 and GD1b; this determinant is formed by the adsorption of GM1 on the GD1b spot during incubation with soluble GM1 or, conversely, by adsorption of GD1b on the GM1 spot during incubation with GD1b.

A question arose: was the immunostaining observed with GD1b alone (Figure 1D) and that observed with GM1/GD1b (Figure 1E and F) due to the same antibodies or to two distinct populations of antibodies? When the NR fraction of the GM1 column was passed through a GD1b column, both types of immunostaining were retained on the column (results not shown), indicating that a single population of antibodies was involved. Furthermore, when the column was washed with KSCN, the eluted fraction (affinity-purified antibodies) contained both immunoreactivities. The possibility that these antibodies were reacting with a determinant formed by the co-localization of GM1 with GD1b was confirmed using two further experimental approaches. (i) A mixture of the two gangliosides adsorbed on ELISA plates showed higher binding of affinity-purified antibodies in comparison with either ganglioside alone (Figure 2A and B). (ii) GM1 and GD1b spots were overlapped on the TLC plate, and higher binding was observed on the area of overlap (Figure 2C).

Comparison of Figure 1D–F reveals an interesting fact: the GD1b spots show differences in not only staining intensity but also spot thickness. This observation was confirmed by "spot fingerprinting" using affinity-purified antibodies (Figure 3A). In non-treated plates, the immunostaining fit perfectly with the orcinol spot, whereas in GM1-coated plates immunostaining was observed in areas where GD1b was not chemically detected. This result indicated a differential binding behavior in the two situations, which was clearly observed when different amounts of GD1b were spotted on TLC plates and immunostained (Figure 3B and C). In non-treated plates, antibody binding to GD1b required a certain amount of antigen adsorbed to the plate surface (density-dependent binding). In GM1-coated plates, in contrast, a typical hyperbolic binding curve was obtained and the above behavior was not observed. In the areas above and below the observed orcinol spot (Figure 3A and B), it was presumed that less amounts of GD1b were present and that antibodies did not bind to these areas in non-treated plates because they required a certain density of antigen to bind. The adsorption of GM1 in these areas in the GM1-coated plate allowed the binding of antibodies, similar to the binding behavior observed for less amounts of GD1b spotted on the TLC plate (Figure 3B and C).

In view of these findings, additional rabbits with neuropathy were screened for the presence of antibodies reacting with co-localized gangliosides. Using GM1-affinity columns, sera were depleted of classical anti-GM1 antibodies and applied to TLC plates set up to achieve spot overlapping of all gangliosides used as antigens (Figure 4A). Three sera out of a total of 27 showed positive results; that is, increased binding to a GM1/GD1b mixture. One of these sera showed reactivity similar to that of Nx7 (Figure 4B), and another showed additional weaker reactivities with GM1/GT1b and GA1/GD1b (Figure 4C). Further characterization of these three sera showed overall binding behavior similar to that of Nx7.

The biological activity of the novel anti-GM1/GD1b antibodies was studied using an in vitro model of neurotransmitter

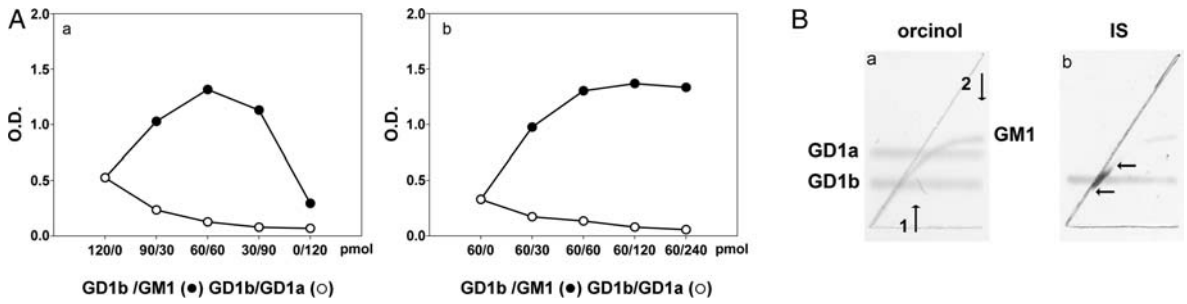

Fig. 2. Antibody binding to co-localized gangliosides. (**A**) Antibody binding to ganglioside mixtures, measured by ELISA. Mixtures of GD1b and GM1 (filled circles) or GD1b and GD1a (open circles) dissolved in methanol were dried on 96-well ELISA plates. Plates were blocked with BSA, wells were incubated with affinity-purified antibodies, and antibody binding was assayed using peroxidase-conjugated goat anti-rabbit IgG. Declining amounts of GD1b and increasing amounts of the ganglioside partner (a), or a fixed amounts of GD1b and increasing amounts of the ganglioside partner (b), were used. (**B**) Antibody binding to gangliosides overlapping on TLC. A mixture of GD1b and GD1a was spotted on TLC plates in a horizontal line (1). After running, the plates were turned upside-down and GM1 was spotted in an oblique line (2). A second run was performed using the same solvent mixture. The developed plates were immunostained with affinity-purified antibodies (IS) or stained with orcinol reagent. The arrows indicate higher staining in the area in which GM1 and GD1b spots overlap.

release (Vilcaes et al. 2009). Affinity-purified antibodies were capable of binding rat synaptosomes (Figure 5A). Additionally, they also inhibited the release of glutamate by a synaptosome suspension (Figure 5B) to a similar degree as that of anti-GM1 antibodies purified from a rabbit that developed neuropathy after immunization with BBG (Moyano et al. 2008).

Discussion

Anti-GM1 IgG antibodies specific to and cross-reactive with GA1 and/or GD1b were induced by the immunization of rabbits with GM1 in a suitable adjuvant (Lopez et al. 2002; Moyano et al. 2008). Similar results were obtained when rabbits were immunized with BBG, despite the fact that BBG contains gangliosides other than GM1. In most cases, anti-ganglioside antibodies retained by a GM1-affinity column were the only ones present in sera (Comín et al. 2006; Moyano et al. 2008). In a few cases (4 of 27 rabbits), an additional immunoreactivity was detected: the serum fraction NR by the GM1-affinity column contained antibodies reacting only with GD1b. These antibodies were purified by affinity chromatography and showed an unusual behavior: their binding was increased when GD1b was co-localized with GM1. This finding suggested that the antibodies recognize a determinant formed by two distinct ganglioside glycans. In ELISA analysis, higher binding was obtained when equal amounts of the two gangliosides were mixed, indicating that the determinant was formed by a pair of molecules. On the other hand, antibody binding to GD1b alone showed "density dependence". This property could be explained as a binding requirement of the antibodies to have an antigenic determinant formed by at least two sufficiently close ganglioside molecules (Nores et al. 1987).

In contrast to classical antibodies that recognize a single antigenic determinant, this new type of antibody recognizes two adjacent (neighboring) determinants of the same or different molecular species. Related to this mechanism, the antibodies display higher affinity because there are more contacts with the antigen. Each binding site of the antibodies can be considered bivalent. Multivalency is a property that is commonly exploited for the reinforcement of binding by proteins that interact with glycans (Chen et al. 2009; Dam and Brewer 2010). The ability of these antibodies to recognize two neighboring antigenic determinants is relevant for glycans because (i) glycans are relatively small molecules, and two of them could fit within the antibody binding site; (ii) glycans form naturally occurring arrays with adjacent molecules, e.g. glycolipids in membranes (rafts), lipopolysaccharides in bacteria and sugar chains in glycoproteins.

Alternatively, we can hypothesize that the new antibodies recognize a ganglioside complex, defined as two neighboring molecules that interact in such a way that they show novel structural determinants not observed in either of each individual molecules. Several recent studies have described antibodies that react with ganglioside complexes (for review, see Kusunoki and Kaida 2011), but there is no direct proof so far of the existence of such complexes.

The controversy between the "neighbor" vs "complex" models cannot yet be resolved, but there is some evidence against the "complex" model. For example, there are no data showing clear side-by-side (*cis*) interaction between ganglioside glycans (Prinetti et al. 2009). In NMR studies of GM1 micelles, carbohydrate–carbohydrate interaction between GM1 monomers was not detected (Brocca et al. 1998). Biophysical studies of gangliosides in monolayers indicate that they can interact laterally with phospholipids and short neutral glycolipids but not with each other (Maggio et al. 1978; Maggio 2004). Sonnino's group recently prepared a dimeric GM1–GD1a hybrid derivative able to bind anti-ganglioside complex antibodies from neuropathy patients (Mauri et al. 2012). These authors concluded from NMR studies that the conformation of the individual gangliosides is maintained in the dimer, although they observed a hydrogen bond between the two terminal galactose residues that could contribute to a more rigid conformation in the dimeric oligosaccharide.

Other recent studies tend to support the "neighbor" model: (i) glycoarrays with differing antigen density revealed distinct reactivities of lectins and antibodies (Oyelaran et al. 2009); (ii) anti-tumor mAbs bound to

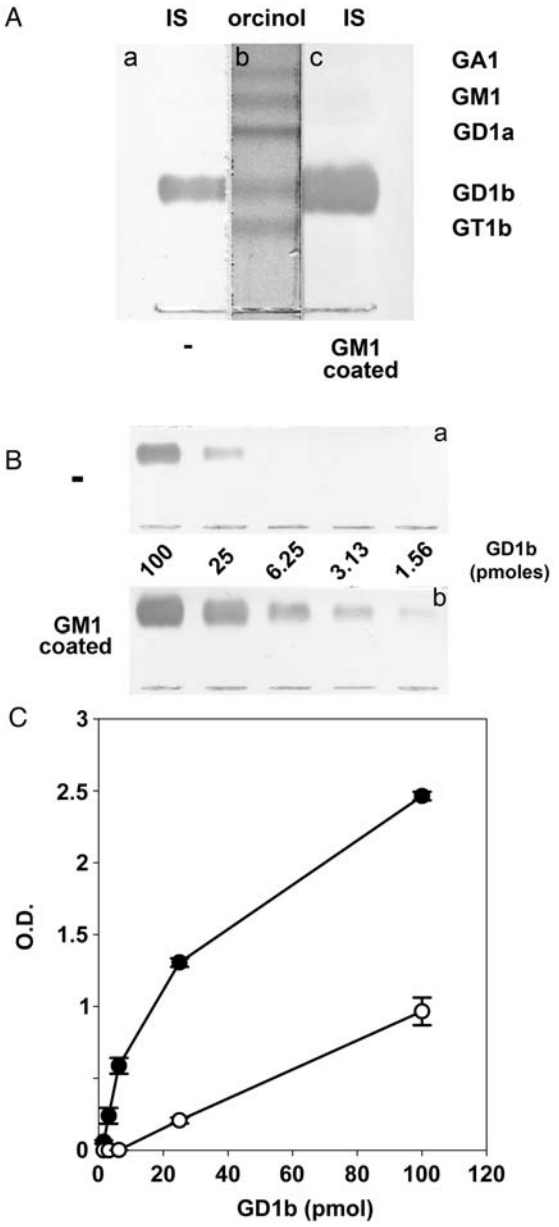

Fig. 3. Binding of antibodies to GD1b alone or to GD1b co-localized with GM1. (**A**) Fitting of orcinol- and antibody-stained spots ("spot fingerprinting"). GD1b was spotted on a TLC plate in a broad straight line. After running, the plate was cut in three strips. The two outside strips were preincubated with GM1 dissolved in PBSt for GM1 coating (c), or with PBSt alone (a), and then incubated with affinity-purified antibodies. The middle strip was stained with orcinol reagent (b). (**B**) TLC immunostaining of various amounts of GD1b in the absence vs the presence of co-localized GM1. (**B**) Various amounts of GD1b as indicated were spotted on TLC plates. After running, the plates were preincubated with GM1 dissolved in PBSt for GM1 coating (b) or with PBSt alone (a), and then immunostained with affinity-purified antibodies. (**C**) Staining intensity was quantified by densitometry. Filled circles, plate preincubated with GM1 in PBSt; open circles, plate preincubated with PBSt alone.

clusters of two or three Tn antigens (α-GalNAc bound to Ser/Thr; Osinaga et al. 2000) and (iii) bivalent antibodies recognizing a virus glycoprotein and other unidentified virus antigens were found in acquired immune deficiency syndrome patients (Mouquet et al. 2010).

Although these new antibodies can be detected and purified by affinity chromatography, one can still question whether they are merely a methodological artifact as opposed to playing any real physiological role. We showed that rabbits immunized with BBG developed a neuropathy (Moyano et al. 2008). The antibodies appeared to be able to target antigens in biological membranes; that is, they bound rat synaptosomes and inhibited their neurotransmitter release. Although this property of the antibodies could have pathological significance, it was not possible to confirm that the antibodies were involved in the development of the disease. All the rabbits that developed the disease, including those with the new type of antibodies, produced classical anti-GM1 antibodies and no differences were detected between rabbits with vs without the new antibodies in terms of clinical symptoms or disease severity.

Experimental procedures

Materials

Total ganglioside fraction was prepared from bovine brain by Folch extraction (Folch-Pi et al. 1957), DEAE-Sephadex chromatography (Yu and Ledeen 1972), alkaline methanolysis and reversed-phase chromatography (Williams and McCluer 1980). This preparation contains four main gangliosides, GM1 (30%), GD1a (48%), GD1b (10%) and GT1b (12%) plus minor components. Individual gangliosides were prepared as described previously (Moyano et al. 2008).

Immunization of rabbits

New Zealand male white rabbits, weight 2–3 kg, were immunized with BBG. For each immunization, 2.5 mg of gangliosides was dissolved in 0.5 mL of phosphate-buffered saline (PBS) containing 1 mg of KLH (Sigma, St Louis, MO) and emulsified in 0.5 mL of complete Freund's adjuvant. The animals were injected subcutaneously on the back and intraperitoneally at 3-week intervals until neurological symptoms appeared. Blood samples were taken by ear vein puncture. Sera were separated from blood clots and frozen at −70°C until use. Several experiments with a total of 27 rabbits were performed. All experiments were performed in accordance with institutional guidelines for animal care.

Enzyme-linked immunosorbent assay (ELISA)

Fifty microliters of a methanolic solution of various concentrations of gangliosides was pipetted into microtiter plate wells and dried overnight at 37°C. Each well was blocked with 1% bovine serum albumin (BSA) in PBS for 1 h, added with 50 µL of BSA-PBS-diluted antibodies, incubated for 4 h and washed with PBS. Binding was detected following 2 h incubation with BSA-PBS diluted (1/1000) peroxidase-conjugated goat anti-rabbit IgG (γ-chain specific; Accurate Chemical & Scientific Corp., NY). All incubation steps were performed at 4°C. After washing, color was developed in a substrate solution containing 15 mM *o*-phenylenediamine and 0.015% H_2O_2 in 0.1 M sodium citrate buffer, pH 5.0, at room temperature. The reaction was stopped after 30 min by

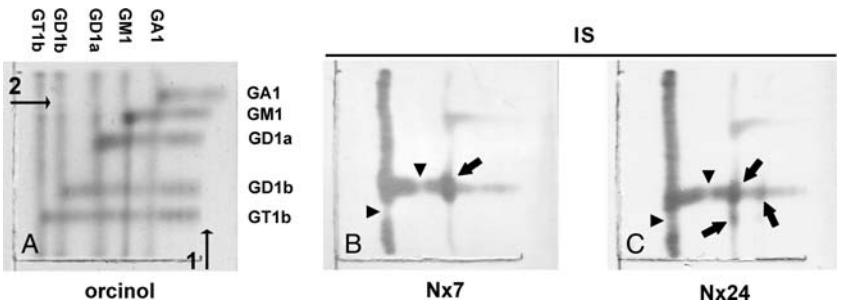

Fig. 4. Antibody binding to co-localized gangliosides. A mixture of gangliosides was spotted on TLC plates in a broad line (1). After running, the plates were rotated 90°, a second ganglioside mixture was spotted, and the plates were run again in the same solvent (2). The plates were then immunostained with the NR fraction of serum. Results from the sera of two representative rabbits are shown: serum (Nx7) showing increased reactivity (arrows) only with co-localized GM1/GD1b (B) and serum with additional reactivity with co-localized GM1/GT1b and GA1/GD1b (C). One plate was stained with orcinol reagent (A).

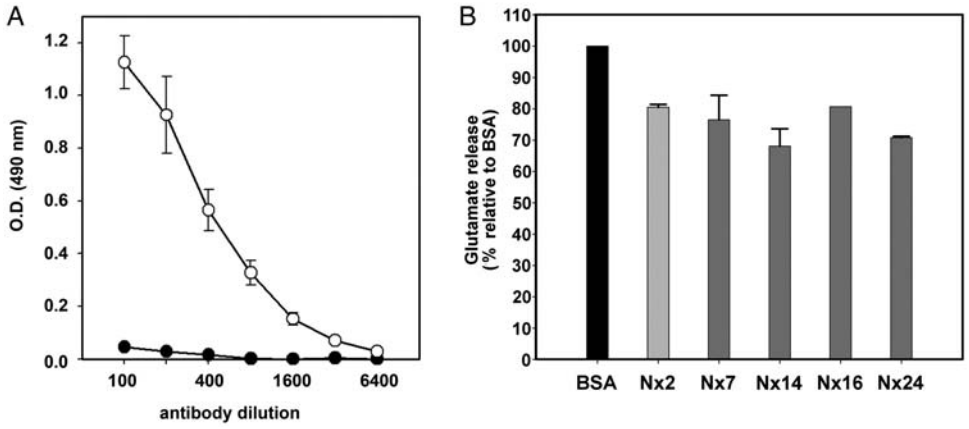

Fig. 5. Effect of antibodies on neurotransmitter release in an in vitro synaptosome model. (**A**) Antibody binding to rat cerebral cortex synaptosomes. Polylysine coated wells with (open circles) or without (filled circles) adsorbed synaptosomes were incubated with serial dilutions of affinity purified antibodies and antibody binding was detected using an ELISA protocol. (**B**) Glutamate release from the synaptosome suspension was induced by 4-aminopyridine as described by Vilcaes et al. (2009). Affinity-purified antibodies were applied to inhibit the release process. Light gray bar: classical anti-GM1 antibodies from a rabbit with neuropathy (Moyano et al. 2008). Dark gray bars: antibodies reacting with co-localized GM1/GD1b from different rabbits. Black bar: control (BSA). Values shown are mean ± SEM from three experiments ($P < 0.01$, Student's t-test).

addition of 100 µL of 0.5 N H_2SO_4, and OD was measured at 490 nm. Non-specific antibody binding (the OD value from a control well without gangliosides) was subtracted from each measured value. Determinations were performed in triplicate.

Thin-layer chromatography immunostaining

A glycolipid mixture containing GA1, GM1, GD1a, GD1b and GT1b was separated on thin-layer chromatography (TLC) plates as described previously (Lopez, Irazoqui, et al. 2000). Plates were air-dried, coated by dipping for 90 s in a 0.5% solution of poly(isobutyl) methacrylate (Aldrich Chemical Co., Milwaukee, WI) in *n*-hexane–chloroform (9:1) and air-dried again for 10 min. For ganglioside coating, plates were incubated for 1 h at 4°C with 0.1 mM GM1 or GD1b in PBSt (PBS containing 0.05% Tween-20) and washed with PBSt. Ganglioside-coated and ganglioside-non-coated plates were blocked with BSA-PBSt for 1 h, incubated overnight in BSA-PBSt diluted serum, washed three times with PBSt, incubated 2 h with peroxidase-conjugated goat anti-rabbit IgG diluted (1/1000) in BSA-PBSt and tested for binding. All incubation steps were performed at 4°C. After washing, color 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), at room temperature. For quantitative studies, spots were measured by scanning densitometry at wavelength 590 nm. In some cases, prior to incubation with plates, diluted serum was pre-incubated (1 h, 4°C) with GM1 at a final concentration of 0.1 mM.

Purification of antibodies on affinity columns

We showed previously that affinity columns prepared by the method of Hirabayashi et al. (1983) are suitable for the isolation of anti-GM1 antibodies (Lopez et al. 2001). About 250 nmol of GM1 or GD1b was bound to 1 mL of octyl-Sepharose (Sigma). Small portions of gel were loaded in small columns and washed with PBS. Rabbit sera (0.25 mL/ mL gel bed) were diluted 1/20 in BSA-PBS and passed through a GM1-column 4×. The non-retained fraction was

used as is or passed through a second column containing GD1b. The GD1b column was washed with PBS and 1 M NaCl. Retained antibodies were eluted with 3 M potassium thiocyanate in PBS, added with BSA, desalted in Sephadex G-25 and frozen at −20°C until use.

Preparation of synaptosomes and glutamate release assay

Cerebral cortex was isolated from Wistar rats, and synaptosomes were purified on discontinuous Percoll gradients as described previously (Dunkley et al. 1986). The synaptosomal pellets were resuspended in 5 mL of HEPES buffer medium consisting of 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.2 mM Na_2HPO_4, 10 mM glucose and 10 mM HEPES, and the protein content was determined by Bradford assay (Bio-Rad, Hercules, CA). The pellets were stored on ice and used within 3–4 h. The total ganglioside fraction of the pellets was similar to BBG; that is, it contained GM1, GD1a, GD1b and GT1b. Glutamate release was assayed by on-line fluorimetry as described previously (Vilcaes et al. 2009).

Synaptosome antibody-binding assay

Fifty microliters of a solution of polylysine (0.1% in 0.1 M borate buffer, pH 8.4) was pipetted into microtiter plate wells and incubated for 12 h at room temperature. After washing with PBS, each well was filled with 50 µL of a suspension of synaptosomes (0.5 mg protein/mL) and incubated for 1 h at 4°C. Wells were washed with PBS, fixed with 4% *p*-formaldehyde in PBS, blocked with BSA-PBS for 1 h and added with 50 µL of BSA-PBS-diluted affinity-purified antibodies. After 4 h incubation, antibody binding was determined using an enzyme-linked immunosorbent assay (ELISA) protocol. Determinations were performed in triplicate.

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Conflict of interest

None declared.

Abbreviations

BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; KLH, keyhole limpet hemocyanin; KSCN, potassium thiocyanate; mAb, monoclonal antibody; HEPES, N-(2-hydroxyethyl)piperazine-N′-ethanesulfonic acid; NR, not retained; OD, optical density. PBS, phosphate-buffered saline; PBSt, PBS containing Tween-20; TLC, thin-layer chromatography.

References

Ariga T, Miyatake T, Yu RK. 2001. Recent studies on the roles of antiglycosphingolipids in the pathogenesis of neurological disorders. *J Neurosc Res*. 65:363–370.

Brocca P, Berthault P, Sonnino S. 1998. Conformation of the oligosaccharide chain of GM1 ganglioside in a carbohydrate-enriched surface. *Biophys J*. 74:309–318.

Chen C, Fu Z, Kim JJ, Barbieri JT, Baldwin MR. 2009. Gangliosides as high affinity receptors for tetanus neurotoxin. *J Biol Chem*. 284:26569–26577.

Comín R, Yuki N, Lopez PHH, Nores GA. 2006. High affinity of anti-GM_1 antibodies is associated with disease onset in experimental neuropathy. *J Neurosci Res*. 84:1085–1090.

Cygler M, Rose DR, Bundle DR. 1991. Recognition of a cell-surface oligosaccharide of pathogenic Salmonella by an antibody Fab fragment. *Science*. 254:442–445.

Dam TK, Brewer CF. 2010. Lectins as pattern recognition molecules: The effects of epitope density in innate immunity (Review). *Glycobiology*. 20:270–279.

Dunkley PR, Jarvie PE, Heath JW, Kidd GJ, Rostas JA. 1986. A rapid method for isolation of synaptosomes on Percoll gradients. *Brain Res*. 372:115–129.

Folch-Pi J, Lees M, Sloane Stanley GM. 1957. A simple method for the insolation and purification of total lipids from animal tissues. *J Biol Chem*. 226:497–509.

Gleeson PA. 1994. Glycoconjugates in autoimmunity. *Biochim Biophys Acta*. 1197:237–255.

Hakomori S, Zhang Y. 1997. Glycosphingolipid antigens and cancer therapy. *Chem Biol*. 4:97–104.

Hirabayashi Y, Susuki T, Susuki Y, Taki T, Matsumoto M, Higashi H, Kato S. 1983. A new method for purification of anti-glycosphingolipid antibody. Avian anti-hematoside (NeuGc) antibody. *J Biochem*. 94:327–330.

Kaida K, Morita D, Kanzaki M, Kamakura K, Motoyoshi K, Hirakawa M, Kusunoki S. 2004. Ganglioside complexes as new target antigens in Guillain-Barré syndrome. *Ann Neurol*. 56:567–571.

Kremer DM, López PHH, Mizutamari RK, Bacile EA, Nores GA. 1997. Factors affecting glycolipid recognition by antibodies: Density-dependent binding of anti-GD1a polyclonal IgG from a neurological patient. *J Neurosc Res*. 47:636–641.

Krengel U, Olsson LL, Martínez C, Talavera A, Rojas G, Mier E, Angström J, Moreno E. 2004. Structure and molecular interactions of a unique antitumor antibody specific for N-glycolyl GM3. *J Biol Chem*. 279:5597–5603.

Kusunoki S, Kaida K. 2011. Antibodies against ganglioside complexes in Guillain-Barré syndrome and related disorders (Review). *J Neurochem*. 116:828–32.

Lopez PHH, Irazoqui FJ, Nores GA. 2000. Normal human plasma contains antibodies that specifically block neuropathy-associated human anti-GM1 IgG-antibodies. *J Neuroimmunol*. 105:179–183.

Lopez PHH, Lardone RD, Irazoqui FJ, Villa AM, Di Egidio M, Saizar RD, Sica REP, Nores GA. 2001. Variable patterns of anti-GM1 IgM-antibody population defined by affinity and specificity in patients with motor syndromes. *J Neuroimmunol*. 119:131–136.

Lopez PHH, Villa A, Sica REP, Nores GA. 2002. High affinity as a disease associated factor in anti-GM1 antibodies: Comparative characterization of experimentally produced vs. disease-associated antibodies. *J Neuroimmunol*. 128:69–76.

Maggio B. 2004. Favorable and unfavorable lateral interactions of ceramide, neutral glycosphingolipids and gangliosides in mixed monolayers. *Chem Phys Lipids*. 132:209–224.

Maggio B, Cumar FA, Caputto R. 1978. Interactions of gangliosides with phospholipids and glycosphingolipids in mixed monolayers. *Biochem J*. 175:1113–1118.

Mauri L, Casellato R, Ciampa MG, Uekusa Y, Kato K, Kaida KI, Motoyama M, Kusunoki S, Sonnino S. 2012. Anti-GM1/GD1a complex antibodies in GBS sera specifically recognize the hybrid dimer of GM1-GD1a. *Glycobiology* 22:351–360.

Mouquet H, Scheid JF, Zoller MJ, Krogsgaard M, Ott RG, Shukair S, Artyomov MN, Pietzsch J, Connors M, Pereyra F et al. 2010. Polyreactivity increases the apparent affinity of anti-HIV antibodies by heteroligation. *Nature* 467:591–595.

Moyano AL, Comín R, Lardone RD, Alaniz ME, Irazoqui FJ, Nores GA. 2008. Validation of a rabbit model of neuropathy induced by immunization with gangliosides. *J Neurol Sci*. 272:110–114.

Murase T, Zheng RB, Joe M, Bai Y, Marcus SL, Lowary TL, Ng KK. 2009. Structural insights into antibody recognition of mycobacterial polysaccharides. *J Mol Biol*. 392:381–392.

Nores GA, Dohi T, Taniguchi M, Hakomori S. 1987. Density dependent recognition of cell surface GM_3 by a certain anti-melanoma antibody, and GM_3 lactone as a possible immunogen: Requirements for tumor-associated antigen and immunogen. *J Immunol*. 139:3171–3176.

Osinaga E, Bay S, Tello D, Babino A, Pritsch O, Assemat K, Cantacuzene D, Nakada H, Alzari P. 2000. Analysis of the fine specificity of Tn-binding proteins using synthetic glycopeptide epitopes and a biosensor based on surface plasmon resonance spectroscopy. *FEBS Lett*. 469:24–28.

Oyelaran O, Li Q, Farnsworth D, Gildersleeve JC. 2009. Microarrays with varying carbohydrate density reveal distinct subpopulations of serum antibodies. *J Proteome Res*. 8:3529–3538.

Prinetti A, Loberto N, Chigorno V, Sonnino S. 2009. Glycosphingolipid behaviour in complex membranes (Review). *Biochim Biophys Acta*. 1788:184–193.

Vilcaes AA, Furlan G, Roth GA. 2009. Inhibition of Ca(2+)-dependent glutamate release from cerebral cortex synaptosomes of rats with experimental autoimmune encephalomyelitis. *J Neurochem.* 108: 881–890.

Vyas NK, Vyas MN, Chervenak MC, Johnson MA, Pinto BM, Bundle DR, Quiocho FA. 2002. Molecular recognition of oligosaccharide epitopes by a monoclonal Fab specific for *Shigella flexneri* Y lipopolysaccharide: X-ray structures and thermodynamics. *Biochemistry.* 41: 13575–13586.

Williams MA, McCluer RH. 1980. The use of Sep-Pak C18 cartridges during the isolation of gangliosides. *J Neurochem*. 35:266–269.

Yu RK, Ledeen RW. 1972. Gangliosides of human, bovine and rabbit plasma. *J Lipid Res*. 13:680–687.

Yuki N, Yamada M, Koga M, Odaka M, Susuki K, Tagawa Y, Ueda S, Kasama T, Ohnishi A, Hayashi S et al. 2001. Animal model of axonal Guillain-Barre syndrome induced by sensitization with GM_1 ganglioside. *Ann Neurol*. 49:712–720.