

Glycolipid antigens and autoantibodies in autoimmune neuropathies

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Autoantibodies to glycans present on glycolipids mediate the postinfectious paralytic disease, Guillain–Barré syndrome (GBS). These glycans are also found on lipooligosaccharides (LOSs) of GBS-inducing microbes, suggesting molecular mimicry as a mechanism for disease induction. How B lymphocyte tolerance to self-glycans is regulated during the initiation phase of the disease is currently under investigation. The discovery of antiglycolipid antibodies that bind to heteromeric glycolipid complexes has generated new insights in this field. Heteromeric complexes are structurally distinct glycolipids that interact to form new molecular shapes capable of either enhancing or attenuating recognition by autoantibodies. Although the principles emerging from this phenomenon have a substantial impact on diagnostics methods, they also raise intriguing questions about the diversity of innate antibody repertoires, mechanisms of tolerance, and autoantibody targeting of neural membranes.

Interface between natural and neuropathogenic antibodies

Antibody repertoires have the capacity to bind a wide range of lipids, glycolipids, and glycans of almost unlimited structural diversity. A large proportion of the human natural antibody repertoire secreted by innate-like B cells contains glycan-binding specificities, both for host and microbial glycans. Indeed 1% alone of the repertoire is estimated to bind the α gal epitope – the Gal α 1-3Gal β 1-(3)4GlcNAc-R saccharide widely expressed in New World monkeys and non-primate mammals, but not expressed in humans, and of key importance in mediating xenotransplant rejection [1]. Although some of these naturally occurring antibodies are often considered polyreactive (i.e., have the capacity to bind structurally dissimilar ligands, albeit with differing affinities and avidities), others appear to be highly specific for discrete glycan epitopes, borne by either glycoproteins or glycolipids [2]. When antiglycolipid antibodies are self-reactive, they have the potential to induce autoimmune disease, notably haemolytic anaemia and neuropathies [3,4]. The apparent incidence of autoimmune neuropathies with an associated autoimmune humoral immune response to glycolipids is increasing as more sensitive assays are

developed, including recently described combinatorial arrays in which antibodies react with heteromeric glycolipid partnerships rather than individual glycolipids [4]. The aim of this review is to describe the origins and specificities of natural antiglycan antibodies in the context of our shifting views on the intricate binding requirements for neuropathy-associated antiglycolipid autoantibodies, away from single oligosaccharides towards heteromeric glycoclusters as antigens. This refocused view of the field opens up new challenges that substantially affect both the design of detection methodologies and our understanding of neurological disease mechanisms driven by autoreactive antiglycolipid antibodies.

Antiganglioside antibodies in autoimmune neuropathy

The prototypic antiglycolipid autoimmune neuropathy is the postinfectious paralytic disorder, Guillain–Barré syndrome (GBS) in which the predominant targets discovered to date are the sialic-acid-bearing glycosphingolipids termed gangliosides. Glycolipids, and in particular gangliosides, were first discovered to be prominent targets for autoantibodies in autoimmune neuropathies including GBS in the 1980s. Around 50 structurally distinct gangliosides are synthesised through stepwise addition of monosaccharides by Golgi glycosyltransferases (Figure 1C) in complex developmental, spatial, and cell-specific patterns [5]. Gangliosides are enriched in neural tissues and primarily localised to domains of the extracellular leaflet of plasma membranes where they are available for antiganglioside antibody binding [6–8]. The first glycolipid molecule to be identified as an IgM autoantibody target was a sulfated glycosphingolipid termed sulfated glucuronyl paragloboside. Shortly afterwards, many other glycolipids and gangliosides including LM1, GM1, GM2, and GD1b were similarly identified as autoantibody targets. Autoimmune neuropathies are a group of heterogeneous inflammatory disorders affecting the peripheral nervous system (PNS) that may be chronic and associated with IgM paraproteinaemia, or acute [9]. GBS is the major acute disorder [10] and foremost cause of postinfectious or post-vaccination neuromuscular paralysis worldwide [11,12]. Three prominent GBS variants occur: acute motor axonal neuropathy (AMAN), Fisher syndrome (FS), and acute inflammatory demyelinating polyneuropathy (AIDP), accounting for ~10%, ~10%, and ~80% of western GBS cases, respectively. Variants are distinguishable clinically, and notably by antiglycolipid antibody profiling. AMAN is closely associated

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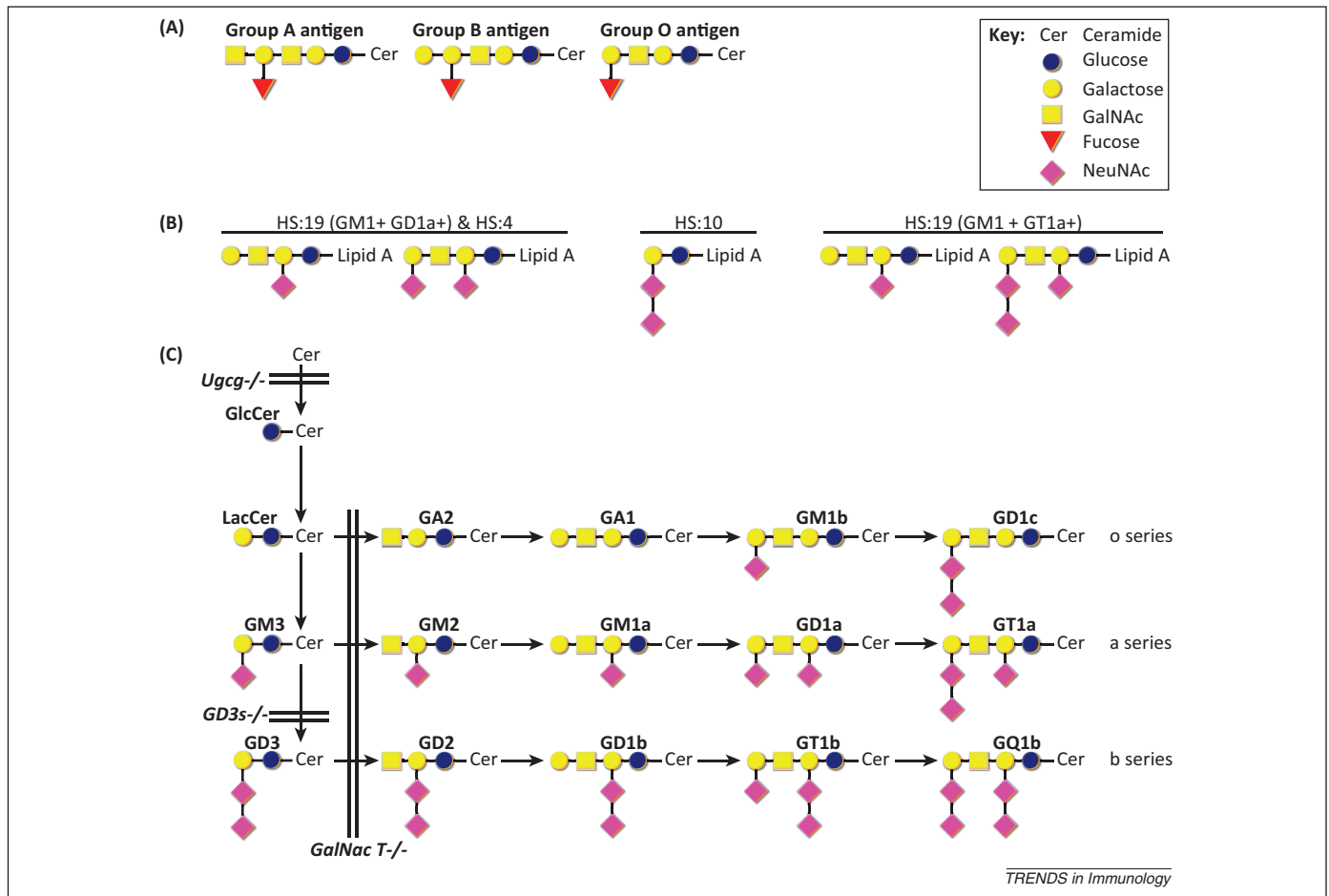


Figure 1. Glycan structures associated with red blood cells, *Campylobacter* lipo-oligosaccharides (LOSs) and membrane gangliosides. **(A)** The ABO blood group antigens are differentiated based on the expression of terminal N-acetylgalactosamine (GalNAc) (Group A), galactose (Group B), or neither (Group O). **(B)** Guillain-Barré syndrome (GBS) associated *Campylobacter jejuni* LOS structures, although linked to lipid A rather than ceramide, are structural mimics of gangliosides. Strains of *C. jejuni* can express multiple structural variants of LOS. **(C)** The biosynthetic pathway of gangliosides is controlled by several glycotransferase enzymes [UDP-glucose ceramide glucosyltransferase (Ugcg), GD3-synthase (GD3s) and GalNAc transferase (GalNAcT)]. Knockout mice for these enzymes (indicated by double lines through the arrows) will not express the subsequent gangliosides.

with antibodies to GM1, GD1a, and GalNAc-GD1a, and FS with antiGQ1b and GT1a antibodies [13–15]. The correlation between antiglycolipid antibodies and AIDP is less well established.

The immunopathogenesis underlying GBS is far from clear, however, it is apparent that a preceding microbial or viral infection or vaccination results in the development of an overt humoral immune response to gangliosides [11]. The classical and most well-studied preceding infection is *Campylobacter jejuni* enteritis [11]. In this acute gastrointestinal infection, individuals generate a predominantly IgA mucosal immune response to *C. jejuni* antigens [16]. Additionally, an estimated 3–12 cases per 10 000 individuals will also generate a transient serum IgG antibody response to self-glycan epitopes also present on microbial LOSs. These epitopes are molecular mimics for gangliosides, which are sialylated glycosphingolipids present in the plasma membranes of all cells within the body. Although the autoimmune response that is generated has the potential to react with gangliosides in all plasma membranes in which they are expressed, the pathological injury that results is restricted to the membranes of peripheral nerves. This specific interaction between antibodies and neural membranes has been attributed to several mechanisms including high

antigen density with low turnover, a relative paucity of endogenous cellular complement inhibitors in neural membranes, and the extreme vulnerability of neurophysiological homeostasis to antibody- and complement-mediated injury [17]. More recently, data describing heteromeric ganglioside complex reactivity suggest that the structural landscape created by interacting glycolipids within the neural plasma membrane partly directs the pathogenesis to that site, and may be uniquely confined to peripheral nerve membranes, which must also be accessible to circulating antibody.

This point is most clearly illustrated by the recent evolution of clinical research centred on glycolipid antibody biomarker discovery. Most previous studies have screened human autoimmune sera against single, purified glycolipids. However, partially overlooked but nevertheless long-standing studies equally have highlighted the importance of accessory lipids and liposomal environments in influencing glycolipid antibody binding [18,19]. Two recent observations have led to a renaissance of interest in this area of multimeric lipid complexes as antibody targets. First, it was discovered that pairs of gangliosides can interact to form heteromeric complexes that enhance binding of neuropathy-associated autoantibodies [20–23]. Second, it was discovered that glycolipid complexes that form naturally in

live nerve membranes can inhibit binding of certain anti-glycolipid autoantibodies to single glycolipids, rendering them pathologically harmless [24]. These enhancing and inhibiting glycolipid complexes also form on solid phase matrices and can thereby be analysed using newly developed immunoassay techniques [25]. Together, these findings indicate that certain classes of antiglycolipid autoantibodies may be far more capricious in their binding behaviour and requirements than is generally perceived.

Natural antibodies to carbohydrate epitopes

Natural antibodies are the product of a range of innate-like B cells that include B1 and marginal zone B cells [26,27]. An over-riding reactivity in this subset of immunoglobulin is to carbohydrate residues, which comprise a normal physiological aspect of the antibody repertoire. They have a range of functions in both organism homeostasis and assistance in the innate and adaptive immune response to infection. A prime example of this anticarbohydrate response is illustrated by the ABO blood group antigens (Figure 1A). This particular reactivity is generated in the first few months after birth and has exquisite specificity and tolerogenic mechanisms in place to deal with this self-glycan antibody response. It is now appreciated that the generation of blood group A- and B-specific IgM and IgA natural antibody response is highly dependent on the gut microbiota [28,29]. Although the B cell repertoire is able to generate both anti-A and -B reactivities, the corresponding inappropriate anti-blood group antigen response is deleted via tolerogenic mechanisms [30]. In general, during an ABO-incompatible blood transfusion the appropriate natural antibody will assist in the rejection of incompatible red blood cells. However, the situation is not as simple during transplantation of ABO-incompatible organs. In this instance the transplant can result in several outcomes depending on the individual [30]. Certain patients will reject based on the overwhelming anticarbohydrate (A or B) response, whereas others who display a robust antibody response will accommodate the transplanted tissue. Finally, a subset of patients who do not generate a significant response are deemed tolerant. However, why certain transplant patients do not respond to the ABO-incompatible blood group antigen is not fully understood. Interestingly, the ABO blood group antigens are terminally fucosylated membrane-associated glycolipids with very similar structures to gangliosides, and similar heterogeneity in tolerogenic control is reflected to a certain extent in GBS. Thus, not all patients who are exposed to *C. jejuni* generate an antiganglioside response. In those that do, the immune response that is generated appears to be due to a transient break in tolerance, which results in an acute autoimmune response directed at the PNS. Why the transient autoreactive response occurs is not known.

Immune environment responsible for a break in tolerance to gangliosides

A wealth of data has shown that, at least in a proportion of GBS cases, crossreactive antiglycolipid antibodies are generated via molecular mimicry during infection, which subsequently leads to a postinfectious nerve injury [31,32]. About two-thirds of GBS patients experience a preceding

upper airway or gastrointestinal infection; the latter being frequently caused by *C. jejuni*. LOSs present on GBS-associated *C. jejuni* isolates contain ganglioside-like structures including GM1, GD1a, GT1a, and GD3, and these are immunologically recognisable by the antiganglioside antibodies in the serum of GBS patients. Thus, antiganglioside antibodies most likely arise through an immune response against *C. jejuni* (Figure 2), and subsequently cause neuropathy by crossreacting with peripheral nerve gangliosides. Notably, infection with *C. jejuni* can lead to a robust immune response to many different bacterial antigens [33], including LOS (Figure 1), but the pathogenesis of *C. jejuni* infection is poorly understood [16]. It has recently been suggested that the pathogenicity is highly dependent on the host intestinal microbiota and innate immune system [34]. Given the right environment, the pathogenicity and invasiveness of *C. jejuni*, which can be enhanced via the presence of sialylated epitopes, induces intestinal epithelial cells to release various factors including chemokine CXC ligand (CXCL)20 and interleukin (IL)-8 [16,35]. This results in the recruitment of haematopoietic cells including dendritic cells (DCs), macrophages, and neutrophils. The subsequent cellular and soluble microenvironment within isolated lymphoid follicles and Peyer's patches leads to a predominately T helper (Th)1 response that can aid in the clearance of bacteria. With regard to GBS, it has been suggested that in the progressive phase of GBS the predominant response is Th1, whereas in the recovery phase this transitions to a Th2 state [36]. Moreover, although in all cases a robust IgA, IgG, and IgM response is generated after *C. jejuni* infection, the ability to overcome tolerance to gangliosides is infrequent and the mechanism unresolved. The big question that thus remains is how the microbiota and inflammation associated with gastrointestinal drives an autoimmune response in susceptible individuals.

The immune system is very tolerant to self-gangliosides, however, in GBS this breaks down and disease ensues, albeit transiently. In mouse models, immunisation with an appropriate LOS generates antibody responses to the structurally similar ganglioside (Figure 1), confirming the existence of immunologically functional mimicry, but overt neurological disease does not follow [37–39]. Humoral responses to LOS species that mimic self-gangliosides are greatly exaggerated in ganglioside-deficient mice compared with wild type mice expressing a normal ganglioside repertoire, thereby confirming that it is tolerance, rather than intrinsic immunological unresponsiveness that regulates antibody generation. In these active immunisation models, documented for GM1, GD1a, and GQ1b gangliosides and their corresponding LOS mimics, the binding of anti-LOS/ganglioside antibodies to peripheral nerve gangliosides in the right context will induce nerve injury via a complement-dependent pathway [17]. Toll-like receptor (TLR)4 is an important endogenous ligand for bacterial LOSs whose activation induces potent inflammatory responses, therefore, it has been widely hypothesised that the interaction of LOSs with TLR4 could play a significant role in driving the maturation of the humoral response to the sialylated epitope displayed on *C. jejuni* LOSs. Analysis of TLR polymorphism in GBS does not show any correlation to disease susceptibility [40]. However, sialylated LOS is more efficient

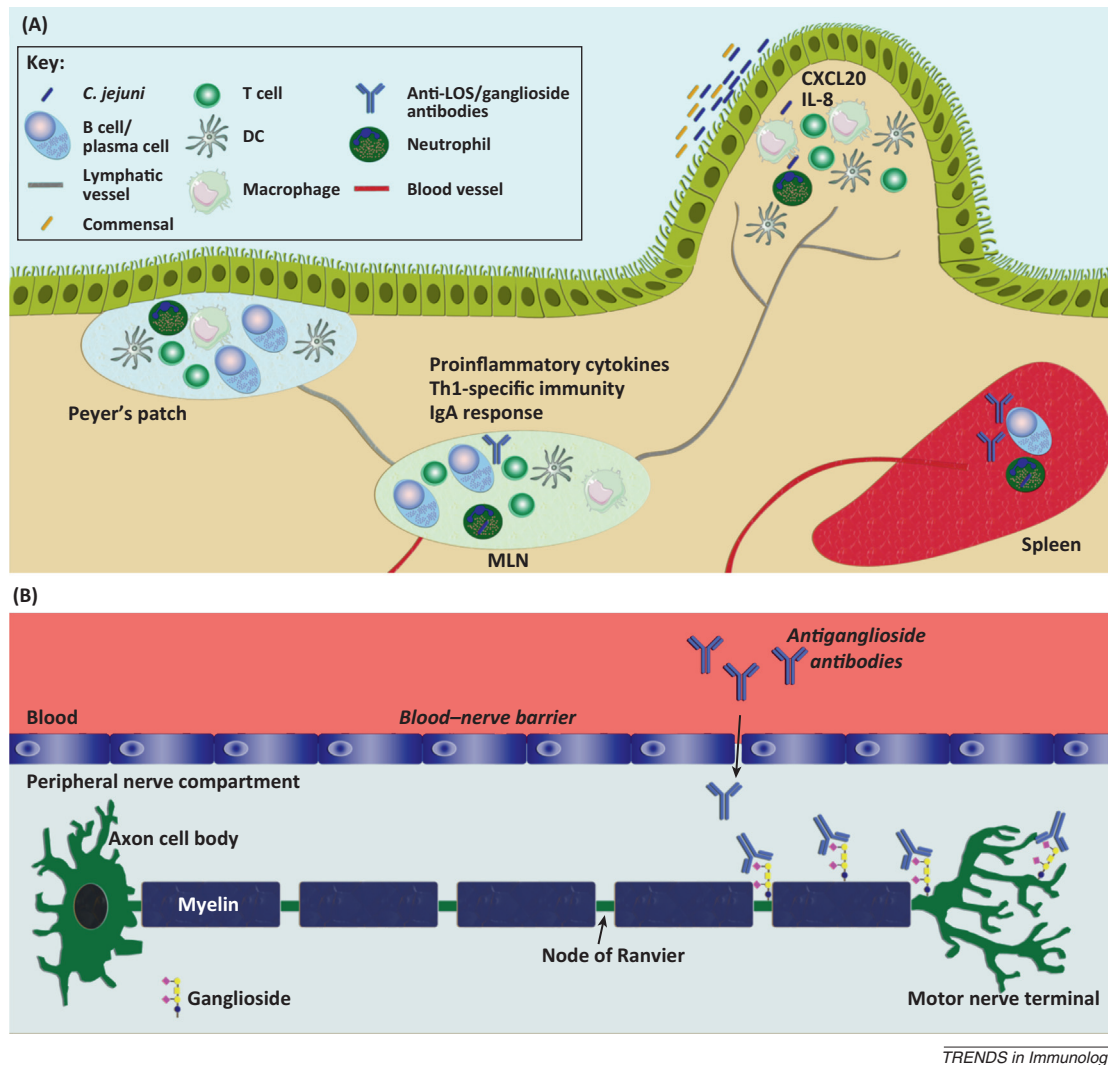


Figure 2. Immune response to *Campylobacter jejuni* and the resulting antiganglioside antibody-mediated pathology. **(A)** Gastrointestinal infection with *C. jejuni* in the context of normal gut microbiota will result in chemokine CXC ligand (CXCL20) and interleukin (IL)-8 secretion by intestinal epithelial cells, with subsequent recruitment of dendritic cells (DCs), macrophages, and neutrophils. Transition of this immune response to the mesenteric lymph nodes (MLNs) or the generation of a response in the Peyer's patches will result in a predominately T helper (Th)1 response for bacterial clearance. Importantly, a robust IgA response is part of this response. It is hypothesised that in individuals who go on to develop GBS, the gut microbiota is altered and that the immune response that is generated to LOS results in an antiganglioside antibody response, which could be due to the contribution of inducible B cell-helper neutrophils (iN_{BH}s) in either the Peyer's patches, MLNs, or spleen. **(B)** The resulting antiganglioside antibody response crosses the blood-nerve barrier and interacts with gangliosides that are expressed on the motor nerve terminal axonal membrane, node of Ranvier, and/or the myelin sheath. Distal nerve injury and injury to the nerve roots may be favoured as these sites lack an effective blood-nerve barrier. Complement is activated, macrophages are recruited, and demyelination and/or axon injury ensues.

at stimulating human DCs and driving the subsequent DC-mediated B cell proliferation than nonsialylated LOS [41]. Moreover, sialylated *C. jejuni* is better at inducing the production of type I interferons via myeloid cells [42], which is known to promote humoral responses in a B cell receptor (BCR)-dependent manner [43]. This has recently been confirmed in studies that have also demonstrated that siglecs, in this case sialoadhesin, play an important role in myeloid cell activation [44].

Antiganglioside responses in GBS patients are generally IgM, IgA, IgG1, and IgG3 [45]. At a mechanistic level the switch to these IgG subclasses implies that T cell help is required. However, it is not understood how glycolipids are presented to the immune system to generate this type of response and studies looking at HLA associations have not in general yielded positive results [46]. Anticarbhydrate antibodies can be generated in a CD1/natural killer (NK)/T

cell-dependent manner, although the extent that this mechanism contributes to the serum antibody response is controversial. In juxtaposition, it has recently been shown that anticarbhydrate responses require the presence of CD4 T cells but not necessarily CD1 and NKT cells [47]. In support of this, modified polysaccharides can induce T cell activation via antigen-presenting cell (APC) processing and presentation on MHCII [48]. Moreover, an alternative MHC-related molecule, MR1 is also able to present lipid [49]. It should also be appreciated that in a T-independent immune response, DCs and neutrophils can take up bacteria and migrate to the spleen, thereby inducing plasmablast differentiation in marginal zone B cells. In the case of neutrophils, a new population has been described in humans, the inducible B cell helper neutrophils (iN_{BH}s), which produce B cell activating factor (BAFF), which aids antiganglioside antibody responses [50], a proliferation-inducing ligand (APRIL),

and IL-21, and are able to prime MZ B cells to secrete IgM [51]. Not only does lipopolysaccharide (LPS) stimulation result in iN_{BH} s, but these cells can also induce class switching and mutagenesis in MZ B cells. The interaction of iN_{BH} s with MZ B cells is thought to generate innate-like defence against microbial infection. However, it is possible that in GBS this drives the breakdown in tolerance. As discussed above, in the context of *C. jejuni*, gastrointestinal inflammation could result in neutrophil/ iN_{BH} recruitment, which would subsequently take up and present antigen (i.e., LOS) once transported to B cell areas [i.e., isolate lymphoid follicles, Peyer's patches, mesenteric lymph nodes (MLNs), and spleen]. This interaction with innate-like B cells could induce nonclassical class switching and the generation of short-lived antiganglioside-antibody-secreting plasma cells. Such a mechanistic explanation still does not explain the transient autoimmune response and the apparent failure to produce a B memory cell compartment.

Antibodies that solely bind to glycolipid complexes

An intriguing conceptual shift in thinking about antiglycolipid antibodies emerged from recent studies [20–23]. In GBS sera that did not harbour any conventionally detectable antibodies to single glycolipids, the authors found categories of antiglycolipid antibodies that only bound to heteromeric complexes of two different glycolipids admixed in 1:1 molar ratios, such as GM1:GD1a. Heteromeric complexes are defined as structurally distinct glycolipids that interact to form new molecular shapes capable of either enhancing or attenuating recognition by autoantibodies (Figure 3), and since this first description a wide range of these have been identified, involving many different heteromeric partnerships. Several models have been

proposed to explain the structural basis for this phenomenon, but none have yet been resolved at the molecular or structural level. Serological studies have demonstrated that anti-GM1:GD1a complex antibodies are capable of binding *C. jejuni* LOS species from organisms expressing GM1- and GD1a-like glycans [52]. Most recently, it was shown that a synthetic hybrid dimer of GM1 and GD1a was also capable of binding anti-GM1:GD1a complex antibodies, thereby strongly suggesting that the antibody binding site involved at least a portion of each oligosaccharide [53].

Local lipid environment within the plasma membrane as a modulator of antibody–glycolipid interactions

The molecular orientation of particular glycolipids within specialised domains in the living plasma membrane may favour the binding of certain antiglycolipid antibodies, yet inhibit the binding of others. Important lateral (*cis*) and *trans* interactions for glycans on cell surfaces that influence lectin binding and signalling have been proposed for many years [54,55]. In the case of anti-GM1 antibodies, the modifying influence of cerebroside, neutral lipids, and cholesterol is long recognised, but had until recently only been studied in artificially created assays, such as liposomes [45,56,57]. An emerging concept that has been proposed argues that heteromeric glycolipid complexes residing in the plane of the plasma membrane form distinctive recognition platforms capable of profoundly influencing autoantibody binding. In one study that examined two anti-GM1 IgG monoclonal antibodies of similar affinities for GM1 (as determined by surface plasmon resonance in solid phase [58]), it was observed that only one was capable of binding GM1 in live plasma membranes, and the other failed to bind GM1 [24]. This dichotomy was also borne out in pathophysiological studies; one being injurious and one being harmless. Subsequent investigation demonstrated that the nonbinding antibody was inhibited from binding GM1 by the adjacent presence in the membrane of GD1a, whereas binding of the other antibody to GM1 was unaffected by GD1a. Subsequently, GBS sera containing the anti-GM1:GD1a complex antibodies described above were examined in the same pathophysiological system and shown to have injurious effects, thereby providing evidence for the existence of a GM1:GD1a complex in the living plasma membrane [59]. Similar types of complex enhanced and inhibited anti-GM1 IgM antibodies have been found in the human disease termed multifocal motor neuropathy [60]. On the one hand the complex can be inhibitory to antibody binding (e.g. GM1/GD1a), and on the other hand the complex acts to enhance the same antibody binding (e.g., GM1/galactocerebroside). Together, these findings indicate that neuropathy-associated antiglycolipid autoantibodies may be complex enhanced, complex inhibited, or indeed complex independent; thus glycolipids *cis*-interact closely with other lipids to form heterogeneous microdomains in living plasma membranes that harbour multicomponent molecular shapes capable of being recognised by antibodies [61,62]. The field is both complicated by, and supported by the fact that many antigen-antibody interactions may be available within the antibody binding site [62].

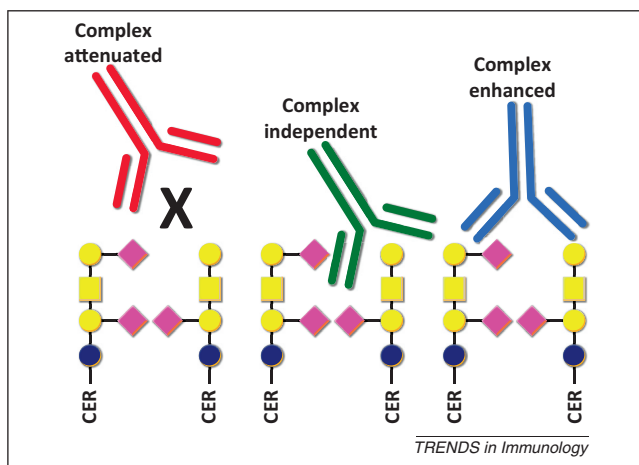


Figure 3. Antibodies that have the capacity to interact with particular gangliosides can have their binding ability influenced by the configuration of other gangliosides with which they laterally interact in the plane of the plasma membrane formed by the local lipid environment. Here this principle is illustrated for heteromeric pairings of GD1a (left) and GM1 (right) ganglioside. In these examples, three categories of anti-GM1 antibody are described. A red anti-GM1 antibody is unable to interact with the ganglioside of choice (i.e., GM1) when a second alternative ganglioside (i.e., GD1a) is present, thereby defined as complex attenuated. A green antibody is able to interact with the GM1 ganglioside even in the presence of another ganglioside, thereby termed complex independent. A blue antibody can only interact with the ganglioside of choice (GM1) when it is present in conjunction with another ganglioside (GD1a), thereby termed complex enhanced. Although these lateral interactions may result in all or no antibody constraints on ligand binding, some antibodies display variable degrees of enhancement and attenuation.

Review

Trends in Immunology xxx xxxx, Vol. xxx, No. x

Antibodies to glycolipid complexes: evolution of new methodologies

Importantly, the number of potential glycolipid ligands is dramatically increased when heteromeric associations are considered, which raises new methodological challenges. For example, from 20 single glycolipids, 190 heteromeric pairings can be generated, making current ELISA techniques impractical, being handling intensive, insensitive, and wasteful of scarce reagents. To address this, a combinatorial glycoarray was developed that miniaturises this heteromeric complex screening procedure into a simple grid format spotted on a polyvinylidene difluoride (PVDF) membrane [25]. Using this method in a screen of GBS sera against 30 lipids, many novel myelin glycolipid complex antigens were identified, which accounted for antiglycolipid autoantibody specificities in >50% of AIDP cases [64]. In a follow-up study on a related anti-GM1 antibody-associated disorder termed multifocal motor neuropathy, antibodies to complexes of GM1:galactocerebroside were identified in otherwise seronegative cases [60]. It is predicted that nerve myelin and/or other Schwann cell membranes and underlying axons may harbour heteromeric, or even multimeric complexes identifiable by antibodies. By building up further complexity in future glycoarray design, it is expected that further anticomplex antibodies will be identified. Definitive studies will require monoclonal antibodies to complexes to investigate this further.

Concluding remarks

In conclusion, the emergence of a class of antiglycolipid antibodies that favour binding to particular and specific clusters of glycans rather than single oligosaccharides heralds a new dawn in autoimmune neuropathy biomarker discovery. Such 'pattern-recognition antibodies' differ substantially from the classical view of antibody binding to a single glycan antigen. Although a substantial amount of progress has been made, some important questions remain, both at the fundamental immunological level and in its translation to human neurological disease. Considering the former, in addition to the explanation for the actual break in tolerance to self-glycans, a major question about the generation of an anticomplex antibody response is precisely how the presentation of LOS with various sialylated epitopes can result in antibodies that interact with neocombinatorial ganglioside epitopes. In terms of human disease, it is important that we determine what are the optimal methodologies for interrogating human neuropathy biobanks for novel antiglycolipid complex autoantibodies, and how can such information be mapped onto the verification of such complexes in living human nerve membranes.

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