STD-NMR Used To Elucidate the Fine Binding Specificity of Pathogenic Anti-Ganglioside Antibodies Directly in Patient Serum[†]

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ABSTRACT: High-resolution binding profiles were elucidated for anti-GM1 IgM autoantibodies from two patients with a progressive form of paraproteinemic polyneuropathy. Antibody−ligand interaction was characterized by generating STD-NMR signals in target ganglio-oligosaccharides added directly to patient sera, without the requirement of antibody fractionation. Both immunoglobulins were found to have similar binding modalities, with interaction confined to two distinct spatially separated regions of GM1: the terminal βGal(1−3)βGalNAc disaccharide unit and the sialic acid residue. We describe a unique and powerful biophysical technique applied to define the molecular interaction between autoimmune disease-causing antibodies and their ganglioside targets.

Autoimmune-associated anti-ganglioside antibodies (Abs)[1] are known to mediate damage to ganglioside-rich nerve cells, leading to both chronic and acute forms of polyneuropathy (PNP) (*1, 2*). Aberrant serum reactivity toward one or a number of gangliosides is most often detected through the use of solid-phase thin-layer chromatography or ELISA measurements. In general, these assays provide estimates of the bulk binding properties of serum Abs toward specific gangliosides, but are poorly descriptive of their fine binding specificity, a reference to the substructural portion of the glycan headgroup that is targeted. Two Abs that are reactive toward a ganglioside, as judged by immunological assays, may have disparate binding propensities for this molecule in a physiological environment because of differences in fine binding specificity (*3, 4*).

Variability in membrane composition among cell types is thought to have important consequences for the structural presentation of constituent glycoconjugate headgroups. This has a direct bearing on the potential physiological effect of an autoantibody, since ganglioside epitopes in some nerve fibers may be Ab-accessible but in others may be cryptic, or nonaccessible. The ability of an Ab to target a particular nerve cell is thought to be a function of both its fine binding specificity and epitope accessibility. Because of the complex nature of the interactions involved, we have a limited understanding of the nature and scope of interactions that characterize pathogenic anti-ganglioside Abs, which limits our ability to understand their pathogenesis.

With these issues in mind, we have undertaken efforts toward the elucidation of atomic-level binding profiles for PNP-associated Abs. As a representative model system, we recently reported epitope maps for purified IgG monoclonal Abs (mAbs) that were raised in ganglioside-deficient mice (*5*). We employed chemoenzymatically synthesized ganglio-oligosaccharides as Ab ligand targets and probed mAb−glycan interaction using saturation transfer difference (STD)-NMR, a technique designed to map protein−ligand interaction (*6*). Disease-causing Abs from PNP patients, however, have proven to be very difficult to affinity purify, and in addition, the quantity of serum required to obtain microgram quantities is prohibitive. Fortunately, STD-NMR by its nature filters out signals from solution components that are not directly involved in protein−ligand interaction. Therefore, ligand binding by authentic pathogenic Abs can potentially be studied using STD-NMR by combining untreated serum directly with ganglio-oligosaccharides.

For our serum-based study, we collected samples from two individuals (designated P1 and P2) diagnosed with a progressive form of paraproteinemic PNP. Immunological screening for anti-ganglioside activity in the patients is reported in Table 1. Both were found to suffer from a monoclonal gammopathy, caused by paraproteins of the IgM class. P1 paraprotein possesses a λ-type light chain with high titers toward gangliosides GM1 and GM2. P2 mAb has a κ-type light chain which is most reactive toward GM1 but also binds GD1b.

When serum from the two patients is spiked with millimolar quantities (∼3−4 mM) of oligosaccharide corresponding to the glycan component of GM1 (termed oligo-GM1), very strong saturation transfer is observed in roughly one-third of the glycan resonances in STD-NMR experiments (Figure 1), delineating the region of contact with the serum Abs. It is important to emphasize that the immunological screen demonstrates that high GM1 reactivity in the sera is due to monoclonal anti-GM1 Abs. It is their presence that

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[1] Abbreviations: Abs, antibodies; PNP, polyneuropathy; ELISA, enzyme-linked immunosorbent assay; NeuAc, *N*-acetylneuraminic acid; STD, saturation transfer difference.

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Table 1: Serum Ganglioside Reactivities and Light Chain Types for Two Patients Suffering from Paraproteinemic PNP

			P1	P2
monoclonal			IgMλ	IgMκ
antibody light chain[a]	GM1	κ	0.05	1.22
		λ	0.49	0.10
	GM2	κ	0.04	−
		λ	0.48	−
anti-ganglioside IgM[b]	GM1		0.74	1.40
	GM2		0.67	0.02
	GD1a		0	0
	GD1b		0.04	0.36
	GD3		0.08	0
	GQ1b		0	0
antibody IgM titers[c]	GM1		3200	25600
	GM2		1600	−
IgM concentration[d]			0.4 g/L	0.5 g/L

[a] Light chain monoclonal gammopathy type determined by immuno-electrophoresis. [b] Anti-ganglioside activity tested by an ELISA and presented as extinctions based on 1:100 dilutions. [c] Serial dilutions used to determine anti-GM1 and anti-GM2 titers. No anti-GM2 serial dilution was performed for P2 sera because of the very low value seen at a 1:100 dilution. [d] Estimated by immunofixation.

gives rise to the saturation signals upon interaction with oligo-GM1 rather than nonspecific interaction with other endogenous serum components. Control experiments with independent batches of serum from healthy individuals spiked with oligo-GM1 did not yield STD signals (Figure 1A). Furthermore, patient sera spiked with control ganglio-oligosaccharides (e.g., GM3-oligo with P1 serum or GM3-oligo and GM2-oligo with P2 serum) did not produce STD signals. In this regard, the NMR and immunological results are fully consistent and complementary.

The STD spectra for P1−oligo-GM1 and P2−oligo-GM1 combinations are similar, indicative of common points of contact between the glycan and mAbs (Figure 1). Fifteen nonequivalent oligo-GM1 protons are saturated as a result of interaction with P1 mAb, while fourteen are saturated due to association with P2 mAb. With the exception of two βGalNAc resonances, all of the oligosaccharide contacts are found in the terminal βGal and NeuAc residues. Of note in comparing the spectra for the two serum samples is the fact that the STD peaks arising from Ab association with the βGal(1−3)βGalNAc disaccharide portion of the molecule are almost identical, not only with respect to the subset of excited protons but also with respect to their relative levels of saturation. This region of the molecule, defined by Ab contact points with eight protons, is clearly a conserved epitope (C-epitope) targeted by both of the autoreactive mAbs. The only observable difference in contact with this region is weak saturation of H2 of GalNAc by P2 mAb, which is not seen with P1.

Almost all differences between the STD spectra for the two patient samples arise as a result of variable Ab association with *N*-acetylneuraminic acid (NeuAc). While there is some P2 mAb interaction with sialic acid, the P1 mAb appears to associate much more extensively with this residue. This is seen, for instance, by comparing the intensity of the NeuAc *N*-acetyl peaks in the two STD spectra, and by the presence of NeuAc H9 and H9′ peaks in the P1 STD spectrum, but not P2 (Figure 1B).

ELISA measurements indicate that in addition to GM1, P1 mAb also reacts with GM2 (Table 1). We acquired STD spectra when oligo-GM2 was added to P1 serum,

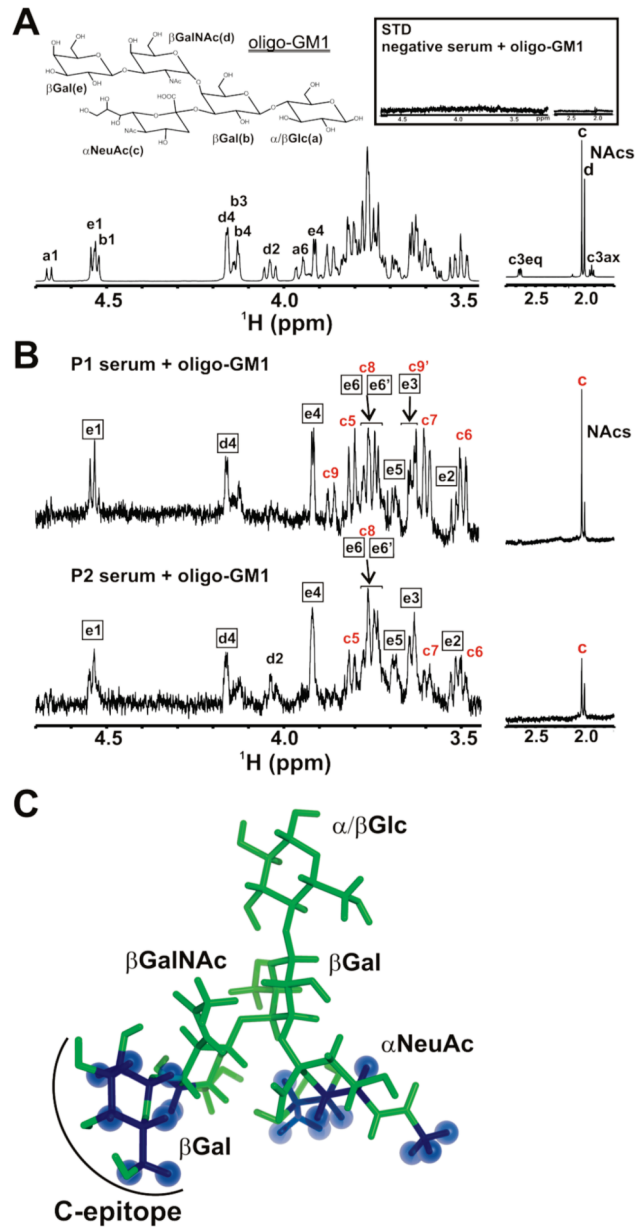

FIGURE 1: Patient mAbs interact with two distinct, spatially separated regions of GM1. (A) Structure of the GM1 glycan and its corresponding ^{1}H spectrum. GM1-oligo added to control serum gives no observable STD signals (inset). (B) GM1-oligo added to P1 and P2 sera gives strong STD signals. Saturated resonances are from the C-epitope (boxed) and NeuAc (red). Signals from sialic acid are much stronger for P1 mAb−GM1 interaction than for P2 mAb. (C) Molecular representation of oligo-GM1. Protons shaded blue correspond to those saturated upon binding with P1 mAb. The C-epitope is almost identically targeted by both patient mAbs.

and the results account for the observed GM1/GM2 cross-reactivity. Eight GM2 resonances are saturated due to interaction with P1 mAb, seven of which are NeuAc protons; the eighth is H4 of βGalNAc (Figure 2). Not surprisingly, the subset of oligo-GM2 sialic acid protons found to interact with this Ab is identical to those mediating its interaction with the analogous residue in oligo-GM1. It is clear that P1 mAb can interact with both of these gangliosides as a result of many close contacts with the NeuAc residue. On the other hand, P2 mAb is not cross-reactive with GM2 (Table 1). This is because there are fewer contacts with the NeuAc residue, as seen in its interaction with oligo-GM1 (Figure 1). There was

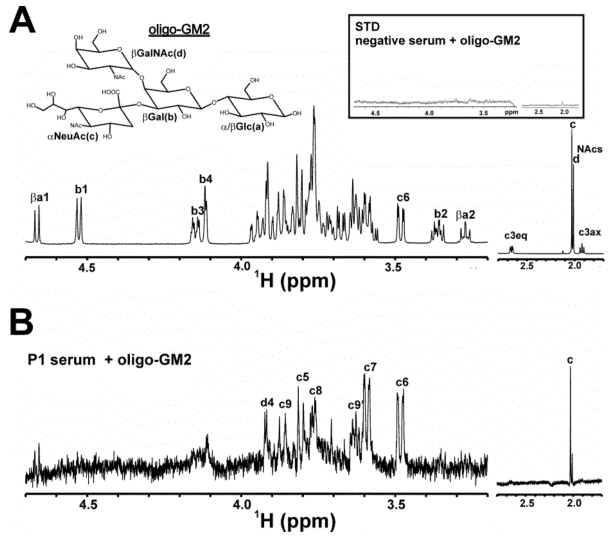

FIGURE 2: P1 mAb cross-reactivity with GM2 is mediated by contacts with sialic acid. (A) Structure of GM2-oligo and its ^{1}H spectrum. No STD signals appear when this oligosaccharide is added to control serum. (B) There are observable STD signals when oligo-GM2 is added to P1 serum. Reactivity arises as a result of many contacts with sialic acid, which accounts for seven of the eight resonances in the STD spectrum. P1 mAb−NeuAc contacts seen with oligo-GM2 match those observed for its interaction with oligo-GM1 (Figure 1).

negligible saturation transfer to oligo-GM2 in STD experiments with P2 serum (data not shown).

To help rationalize the recognition pattern of the Abs, we constructed a molecular representation of oligo-GM1 based on published glycosidic dihedral angles (*7*). GM1 oligosaccharide forms a triangular-shaped structure. Coloring of its protons saturated in serum STD experiments demonstrates that patient mAb binding occurs at spatially separated regions, localized at two outer edges of the triangular arrangement (Figure 1C). As noted above, the STD signals arising from contact with the terminal βGal(1−3)βGalNAc disaccharide are practically identical in both cases, indicative of a common structural motif accessed by the two immunoglobulins.

The binding profiles displayed by the Abs are reminiscent of the interaction of GM1 with cholera toxin, which also targets the βGal(1−3)βGalNAc disaccharide and the NeuAc residue in what has been termed a "two-fingered grip" (*8*). Bivalency is believed to impart a high level of specificity to the formation of the toxin−GM1 complex and may be an important contributing factor to the strong binding affinity governing protein−carbohydrate interaction (*9*). The close resemblance in the mechanism of GM1 binding by the mAbs and cholera toxin suggests there may be a limited subset of potential protein−GM1 contacts within a membrane environment. Although their binding modalities are very similar, subtle differences give rise to altered binding characteristics, such as their ability to cross-react with related gangliosides (Table 1).

In conclusion, we have successfully epitope-mapped two disease-causing anti-GM1 IgM Abs from PNP patients, without prior fractionation from other serum components. While the general applicability of our strategy needs to be further explored with additional protein−ligand systems, our results indicate that STD-NMR offers the potential to investigate ligand binding by serum-based proteins for which purification and subsequent characterization is not tractable.

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SUPPORTING INFORMATION AVAILABLE

Protocols for oligosaccharide synthesis, immunological serum screening, and STD-NMR acquisition, as well as NMR assignments for oligo-GM1 and oligo-GM2. This material is available free of charge via the Internet at http://pubs.acs.org.

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