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Molecular Analysis of Carbohydrate−Antibody Interactions: Case Study Using a *Bacillus anthracis* Tetrasaccharide

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Abstract: The process for selecting potent and effective carbohydrate antigens is not well-established. A combination of synthetic glycan microarray screening, surface plasmon resonance analysis, and saturation transfer difference NMR spectroscopy was used to dissect the antibody-binding surface of a carbohydrate antigen, revealing crucial binding elements with atomic-level detail. This analysis takes the first step toward uncovering the rules for structure-based design of carbohydrate antigens.

Carbohydrate-based vaccines, which induce an immune response against cell-surface oligosaccharides found on disease-causing bacteria, are now used to protect young children from meningitis and other severe bacterial diseases.[1−4] However, selecting an oligo- or polysaccharide antigen that will generate highly selective antibodies is not a straightforward process, and the rules have not yet been defined. Typical oligosaccharides contain few, if any, charged residues and primarily display only hydroxyl and amine groups on their sugar rings. This starkly contrasts with the rich structural and chemical diversity of peptides, which are better-understood antigens. Understanding how complex carbohydrates interact with antibodies is an important first step toward establishing rules for guided carbohydrate antigen design.[5] After the pioneering work by Lemieux,[6] the antibody response to immune challenge with antigens from *Salmonella*,[7] *Shigella flexneri*,[8] *Vibrio cholerae*,[9] and *Candida albicans*[10] has been investigated. Since crystallization is rarely possible, enzyme-linked immunosorbent assay (ELISA)-based formats, immunoblotting, and surface plasmon resonance (SPR) analysis have been utilized to define key epitopes. Here we show that crucial antibody-binding positions on the sugar antigen can be identified using a combination of three established techniques: synthetic glycan microarray screening, SPR analysis, and saturation transfer difference (STD) NMR spectroscopy. It is the combination of these complementary techniques that reveals the molecular interactions between antibodies and carbohydrate antigens. We have examined the tetrasaccharide component of the glycoprotein Bacillus collagen-like protein of anthracis (BclA), the highly immunogenic glycoprotein found on the surface of the spores of *Bacillus anthracis*, the agent that causes the acute zoonotic disease anthrax. This tetrasaccharide was a particularly attractive target because glycans found on the *B. anthracis* spore surface are excellent candidate antigens for developing anthrax vaccines and diagnostic tools.[11,12]

The BclA tetrasaccharide contains three rhamnose residues and an unusual terminal sugar, 2-*O*-methyl-4-(3-hydroxy-3-methylbutanamido)-4,6-dideoxy-D-glucopyranose, which is named anthrose.[13] This tetrasaccharide and related structures were synthesized chemically and successfully used as both antigens and molecular probes to identify *B. anthracis*.[14−18] Furthermore, synthetic tetrasaccharide **1** (Figure 1), was immunogenic in mice and generated the monoclonal antibodies (mAbs) MTA1−3.[19,20] The selectivity of the anti-tetrasaccharide and anti-disaccharide mAbs was shown by immunofluorescence analyses, where the antibodies distinguished *B. anthracis* spores even from those of closely related strains such as *Bacillus thuringiensis*.[19−21] For the purposes of this study, anti-disaccharide mAbs (designated MTD1−6) were obtained by B-cell hybridoma technology using spleen cells of mice immunized with the BclA-related disaccharide **15** (Figure 1).

To uncover which structural and chemical elements of the carbohydrate influenced this selectivity, microarray screening was performed using a family of synthetic oligosaccharides related to the original BclA tetrasaccharide. The synthetic oligosaccharide analogues ranged from mono- to tetrasaccharides and were equipped with different side chain appendages as well as a thiol-modified linker at the reducing end for attachment to a maleimide-functionalized microarray.[22] These synthetic glycans were screened for their abilities to bind the anti-disaccharide mAbs (MTD1−6) and the anti-tetrasaccharide mAbs (MTA1−3) (Figure 1). The anti-tetrasaccharide and the anti-disaccharide mAbs exhibited profoundly different binding patterns. The anti-disaccharide mAbs recognized all of the synthetic structures with intact anthrose, including anthrose monosaccharides (**1**−**6** and **12**−**16**). Similarly, the anti-tetrasaccharide mAbs strongly bound tetrasaccharide analogues **1** and **2** and trisaccharide **16**. However, the anti-tetrasaccharide antibodies only weakly bound tetrasaccharide analogues **3**, **4** and **6**, and tetrasaccharide analogues **5** and **7** were not bound at all. Notably, each of these structures contained a modified terminal anthrose. None of the antibodies (anti-disaccharide or anti-tetrasaccharide) recognized mono-, di-, or trirhamnose structures (**8**−**11**). Altogether, these results demonstrate that anthrose is the minimal unit required for binding anti-disaccharide mAbs. Interestingly, while a terminal anthrose is absolutely required for oligosaccharide recognition by the anti-tetrasaccharide mAbs, these mAbs failed to bind the anthrose-containing truncated mono- and disaccharide structures

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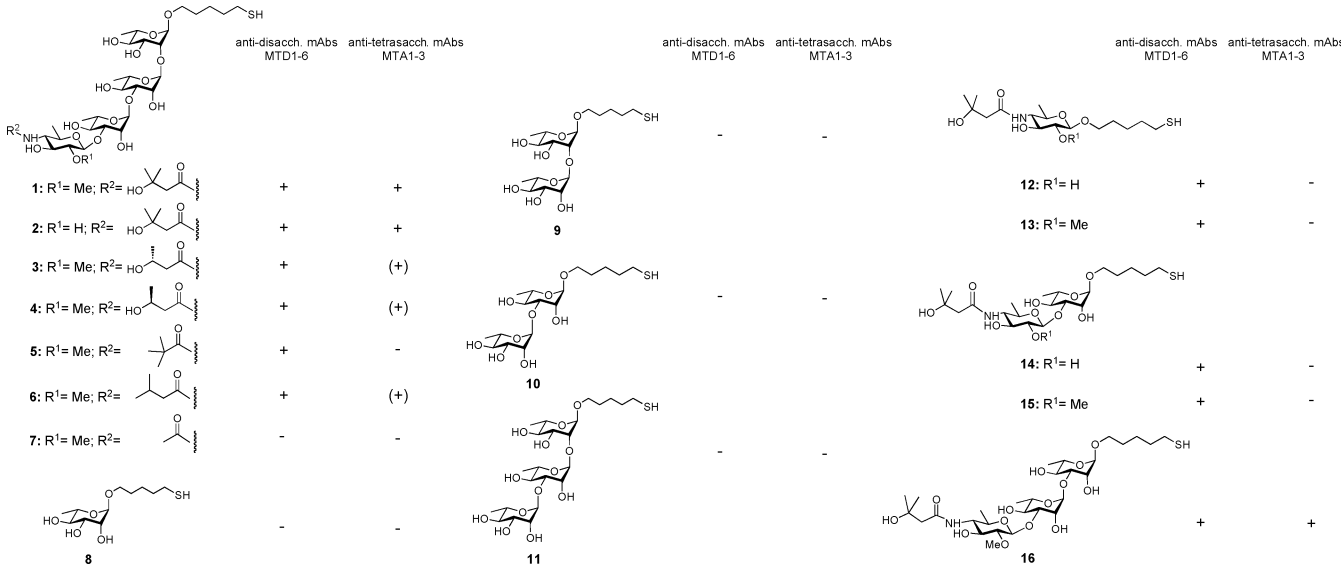

Figure 1. Synthetic glycans related to the *B. anthracis* cell-surface tetrasaccharide BclA. The synthetic glycans were used for antibody mapping by microarray screening, SPR, and STD NMR analyses. Microarray analysis demonstrates the cross-reactivity of monoclonal antibodies generated against anthrose−rhamnose disaccharide **15** (MTD1−6) and tetrasaccharide **1** (MTA1−3).

12−**15.** Therefore, the anti-tetrasaccharide mAbs require at least two rhamnose units as well as the terminal anthrose for tight oligosaccharide binding.

Many glycans, particularly in mammalian systems, do not contain side chain appendages, but anthrose does. Since the anthrose sugar was essential for antibody binding, its distinctive side chain was investigated in greater detail. A drastic truncation of the chain produced by reducing 3-hydroxy-3-methylbutyrate to acetate (**7** in Figure 1), resulted in a structure that was not recognized by any mAb. However, deleting a methyl group within the side chain by replacing 3-hydroxy-3-methylbutyrate with 3-hydroxybutyrate (**3** and **4** in Figure 1) reduced binding of the anti-tetrasaccharide mAbs dramatically but had little effect on binding anti-disaccharide mAbs. Similarly, placement of a trimethylacetyl moiety (**5** in Figure 1) or deletion of a 3-hydroxyl group (**6** in Figure 1) significantly affected only anti-tetrasaccharide mAb binding. Therefore, while the anthrose side chain must be present for the glycan to bind both classes of mAbs, only the anti-tetrasaccharide mAbs are affected by altering the specific chemical composition of the side chain, such as removing the C3 methyl group.

Quantification of the carbohydrate−antibody binding interactions commenced using SPR analysis, which reconfirmed the tight interaction (K_D = 9.1 μM) between the anti-tetrasaccharide mAb MTA1 and its original tetrasaccharide antigen [Table 1 and Figure 2 in the Supporting Information (SI)]. SPR analysis also demonstrated that this interaction is fast, indeed much faster than binding of anti-disaccharide antibodies with any ligand (SI Table 1 and SI Figure 2). Consistent with the microarray results, MTA1 did not bind with significant affinity any of the other synthetic glycans tested (SI Table 1). The anti-disaccharide mAb MTD6 showed unusually high affinity for its original disaccharide antigen (K_D = 0.51 μM; SI Table 1). Few carbohydrate−antibody interactions with K_D values less than 1 μM have been reported, which makes this discovery particularly significant. Interestingly, the K_D values were comparable for interactions between MTD6 and two structurally diverse oligosaccharides, the tetrasaccharide (K_D = 3.7 μM) and the anthrose monosaccharide (K_D = 7.2 μM) (SI Table 1 and SI Figure 2). On the basis of this small difference in K_D, we can conclude that the rhamnose units in the tetrasaccharide contribute little to binding MTD6.

Unfortunately, the extremely slow dissociation of the MTD6−disaccharide complex prevented further analysis of this antibody-oligosaccharide pair using STD NMR spectroscopy.[23,24] This method is particularly suited for characterizing binding differences within ligands (discriminating tightly bound domains from weakly bound ones) without having to assign the resonances of the macromolecular receptor. However, slow kinetics results in very limited transfer of ligands from the antibody-bound state to the free state, strongly affecting the signal-to-noise ratio in STD NMR experiments. In addition, an increased antibody−ligand complex lifetime results in intraligand spin diffusion that decreases the discrimination between individual positions of the ligand and prevents detailed epitope mapping. The complex of MTA1 and the tetrasaccharide, however, was a good candidate for further analysis using STD NMR spectroscopy. Assessment of antibody binding at a 30:1 ratio of carbohydrate ligand to protein confirmed that MTA1 tightly bound all four sugars of the tetrasaccharide (Figure 2) but had little effect on the unnatural linker at the reducing end of rhamnose D. Strong STD effects indicated that tight-binding sites were located throughout the entire tetrasaccharide on all four sugars, with a cluster of tight-binding sites found within the anthrose−(β1−3)−rhamnose substructure (Figure 2). Binding was relatively weaker at the opposite end of the molecule, but STD effects showed that binding in this region was still significant (Figure 2). Looking at STD effects throughout the structure, we observed that one face of the trirhamnose chain (protons H1−H2−H3) was bound more tightly by the antibody than the opposite side (protons H4−H5−H6). The H1−H2−H3 face of the trirhamnose chain is likely to be oriented closer to the antibody within the tetrasaccharide−antibody complex. STD analysis had indicated that within the anthrose unit there is a cluster of sites tightly bound by MTA1. Specifically, on the anthrose sugar ring, three protons showed strong STD effects, but the 2″-*O*-methyl group was bound less strongly. This observation agrees with our microarray data, which indicated that this side chain appendage was of minor importance for recognition by MTA1 (**1** and **2** in Figure 1). The microarray data also demonstrated that the anthrose C4 side chain was crucial for binding MTA1, and this finding is supported by the STD NMR results, which showed that within the C4 chain, a methylene group as well as two methyl groups appear to have

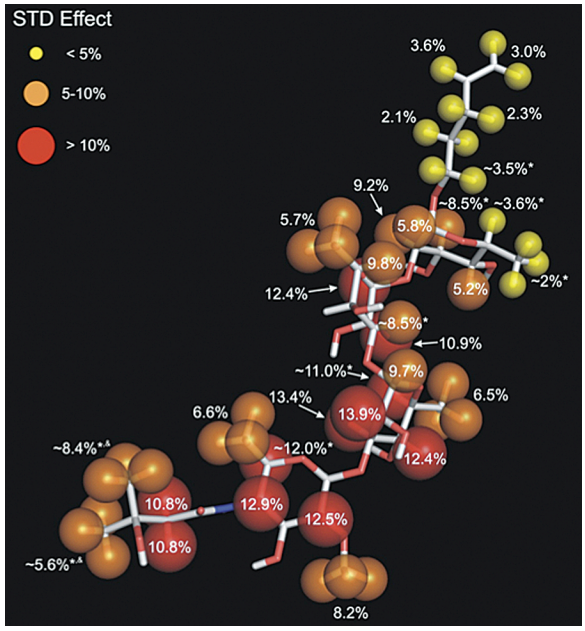

Figure 2. Epitope mapping of the BclA tetrasaccharide **1**−MTA1 interaction by STD NMR spectroscopy. Percent STD effects are shown for individual protons of tetrasaccharide **1**. In addition, strong (>10%), medium (5−10%), and weak (<5%) STD effects are indicated by red, orange, and yellow spheres of decreasing size. Positions marked with an asterisk could not be determined with high accuracy because of resonance overlap. The methyl groups marked with & superscripts were not assigned stereospecifically.

significant STD values (Figure 2). Interestingly, the two methyl groups have different STD values, indicating that one methyl group is oriented closer to MTA1 and is bound more tightly. It is remarkable that this difference in binding affinity was also detected by microarray screening, where tetrasaccharide **4** containing a 4-(3*S*)-3-hydroxy-3-methylbutyrate side chain showed stronger affinity toward an anti-tetrasaccharide mAb, MTA2, than did **3**, which is decorated with a 4-(3*R*)-3-hydroxy-3-methylbutyrate side chain (SI Figure 1). We therefore conclude that the methyl group presented in the *S* enantiomer, **4**, is proximal to the antibody and thus makes a greater contribution to binding.

The power of systematically combining microarray profiling, SPR analysis, and STD NMR spectroscopy has been revealed in this study, where we precisely mapped the molecular elements of the BclA tetrasaccharide that participate in tight antibody binding. Understanding which structural features of the oligosaccharide are most important for this interaction will enable the design of better carbohydrate-based anthrax vaccines. Furthermore, this study has illuminated the binding requirements for mAbs that are currently under development as a highly sensitive spore detection system.[25] The approach, however, is a more general tool that we hope could ultimately help to elucidate the general principles of carbohydrate−antibody interactions, enabling guided structure-based design of a broad spectrum of carbohydrate-based antigens and therapeutics.

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Supporting Information Available: Experimental procedures, characterization data for new compounds, supporting figures and tables, and complete ref 2 (as SI ref 7). This material is available free of charge via the Internet at http://pubs.acs.org.

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