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RAPID COMMUNICATION

Photogenerated glycan arrays identify immunogenic sugar moieties of *Bacillus anthracis* exosporium

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Using photogenerated glycan arrays, we characterized a large panel of synthetic carbohydrates for their antigenic reactivities with pathogen-specific antibodies. We discovered that rabbit IgG antibodies elicited by *Bacillus anthracis* spores specifically recognize a tetrasaccharide chain that decorates the outermost surfaces of the *B. anthracis* exosporium. Since this sugar moiety is highly specific for the spores of *B. anthracis*, it appears to be a key biomarker for detection of *B. anthracis* spores and development of novel vaccines that target anthrax spores.

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Anthrax is a fatal infectious disease caused by the Gram-positive, rod-shaped bacterium *B. anthracis*. Anthrax infection is initiated by the entry of spores into the mammalian host *via* intradermal inoculation, ingestion, or inhalation [1]. The most lethal form of human anthrax is the pulmonary infection caused by inhaled spores. In view of the risk of *B. anthracis* spores as a biological weapon of mass destruction (WMD) [2], it is necessary to achieve the capacity for the rapid and specific detection of *B. anthracis* spores in various conditions [3, 4]. It is also important to develop new vaccines to block the anthrax infection at its initial stage before spore germination takes place [5, 6]. In this context, identification of highly specific immunogenic targets that are displayed on the outermost surfaces of *B. anthracis* spores is of utmost importance.

Substantial effort has been made to study the structure and antigenic elements of the outer layers of *B. anthracis* spores. The mature *B. anthracis* spore contains a central genome-containing core compartment and three adjacent protective layers: the cortex, coat, and exosporium [7–9]. The exosporium is at the outermost surface and is fully exposed to the external environment. Morphologically, the exosporium has two distinct structures, a paracrystalline basal layer and an external hair-like nap [8]. Isolated *B. anthracis* exosporia contain as many as 20 protein components [9, 10]. The most prominent element is the BclA (for *Bacillus* collagen-like protein of *anthracis*) glycoprotein [11, 12].

The BclA glycoprotein displays a unique rhamnose-containing tetrasaccharide. This carbohydrate moiety is capped at its upstream end with a previously unknown sugar residue termed anthrose [2-*O*-methyl-4-(3-hydroxy-3-methylbutamido)-4,6-dideoxy-β-D-glucose] [12]. The complete structure of this tetrasaccharide has been determined to be 2-*O*-methyl-4-(3-hydroxy-3-methylbutamido)-4,6-dideoxy-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→3)-α-L-

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Abbreviation: BclA, *Bacillus* collagen-like protein of *anthracis*

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rhamnopyranosyl-(1→2)L-rhamnopyranose [12]. Importantly, this sugar moiety is absent on the surfaces of *B. anthracis* vegetative cells or the spores of other bacillus strains, including *B. cereus* and *B. thuringiensis*, the two strains phylogenetically most close to *B. anthracis* [12].

Surface-exposed carbohydrate moieties that are characteristic of a given microbe may serve as key biomarkers for pathogen identification, diagnosis, and vaccine development. Therefore, the anthrose-containing tetrasaccharide of the BclA glycoprotein is an attractive target for immunological investigations. BclA has been confirmed to be immunodominant [13]. However, whether its carbohydrate moieties contribute to the immunogenecity of this exosporium glycoprotein remains unknown [14]. This may be due to the lack of a method for the detection of antibodies specific for the carbohydrate moieties of the glycoprotein. It has, therefore, been difficult to decipher the anticarbohydrate specificities and antiprotein specificities, even if the corresponding antibodies were present in the sera as a result of a polyclonal antibody response to a natural infection or a vaccination using immunogenic glycoconjugates.

We present here a high-throughput strategy to facilitate identification and immunological characterization of pathogen-specific carbohydrate moieties, including those of the *B. anthracis* exosporium. This strategy employs highly sensitive oligosaccharide microarrays to probe specific antibodies that were elicited by infections or immunizations. Our rationale was that if a pathogen expressed immunogenic carbohydrate structures, then immunizing animals using this microbe or its antigen preparations would have the possibility to elicit antibodies specific for these structures. Characterizing these antibody responses using a broad-range glycan array that displays the corresponding saccharides may thus rapidly identify the immunogenic sugar moieties of the corresponding pathogens. A schematic overview of this biomarker identification strategy is shown in Fig. 1.

Photogeneration of epitope-specific glycan arrays is a key component of this strategy. We utilized a photoactive surface for covalent immobilization and micropatterning of carbohydrates onto glass substrates [15]. This method employs a glass slide coated with a self-assembled mixed monolayer that presents photoactive phthalimide chromophores at the air–monolayer interface [15]. Upon exposure to UV radiation (300 nm), the phthalimide end-groups graft the spotted carbohydrates by hydrogen abstraction followed by radical recombination. The efficacy of carbohydrate immobilization is independent of the molecular weights of spotted carbohydrates [15]. Thus, a unique technical advantage of this method is the ability to produce epitope-specific glycan arrays using unmodified mono- and oligo-saccharides. We applied, therefore, this technology to display a large panel of saccharide structures, including synthetic fragments and derivatives of the anthrose-containing tetrasaccharide side chain of the *B. anthracis* exosporium [12, 16–18] and a number of control carbohydrate antigens (Supplementary Table 1), for an immunological characterization.

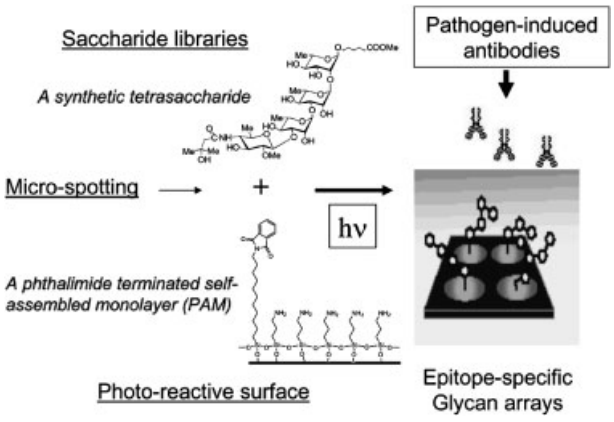

Figure 1. Photogenerated glycan arrays for rapid identification of pathogen-specific immunogenic sugar moieties. Saccharide preparations were dissolved in saline (0.9% NaCl) at a given concentration and spotted using a high-precision robot (PIXSYS 5500C, Cartesian Technologies Irvine, CA) onto the phthalimide-amine monolayers (PAM)-coated slides. The printed PAM slides were subjected to UV irradiation (300 nm) for 1 h to activate the photocoupling of carbohydrates to the surface. Pathogen-specific antisera were then applied on the glycan arrays to identify potential immunogenic sugar moieties of given pathogens.

At the onset of this work, we assumed that if *B. anthracis* spores express potent immunogenic carbohydrate moieties, immunization with the spores would elicit antibodies specific for these sugar structures. Such antibody reactivities would then be detected by glycan arrays that display the corresponding sugar structures. Therefore, we incubated the anthrax saccharide glycan arrays with rabbit anti-*B. anthracis* spore antibodies and examined the potential presence of anticarbohydrate specificities.

Figure 2 shows a glycan array image that was stained with a preparation of pooled rabbit polyclonal antianthrax spore IgG antibodies. Images (a-f) display a portion of the stained glycan arrays in the absence or presence of saccharide inhibitors: (a) no saccharide inhibitor; (b) anthrose; (c) D-glucose; (d) α-anthrose disaccharide; (e) α-anthrose tetrasaccharide; (f) β-anthrose tetrasaccharide. As highlighted with colored boxes in the image, a number of immobilized carbohydrate probes detected significant amounts of IgG antibodies. These include an anthrose-containing trisaccharide (White, array location B-1), and both the β-tetrasaccharide (Brown, array location C-1) and α-tetrasaccharide (Yellow, array location B-4) in the form of their methyl 6-hydroxyhexanoyl glycosides. The glycan array also shows other anticarbohydrate antibody activities, such as antipolysaccharide antibodies for isolichenin [α(1,3)glucan] (positive spots in the upper-left corner) and *Streptococcus pneumoniae* type 23 (Pn 23) polysaccharide (positive spots in the bottom-right corner). These reagents were spotted on the chips as positive controls for the assay system since we detected a significant amount of IgG antibodies for these antigens in this preparation of rabbit polyclonal IgGs in a preliminary experiment.

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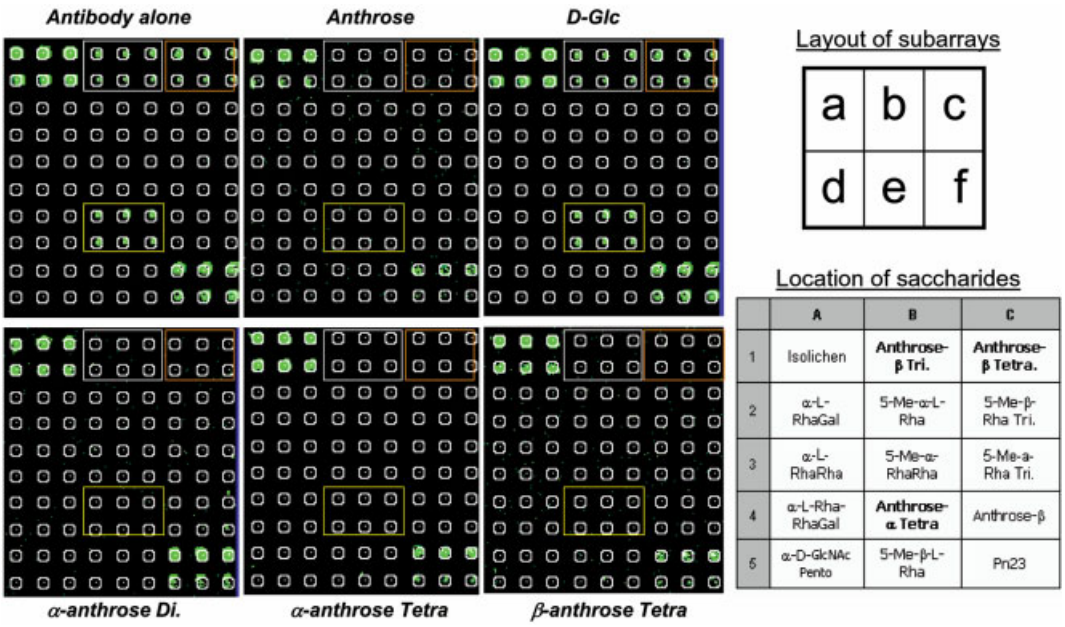

	A	B	C
1	Isolichen	Anthrose-β Tri.	Anthrose-β Tetra.
2	α-L-RhaGal	5-Me-α-L-Rha	5-Me-β-Rha Tri.
3	α-L-RhaRha	5-Me-α-RhaRha	5-Me-a-Rha Tri.
4	α-L-Rha-RhaGal	Anthrose-α Tetra	Anthrose-β
5	α-D-GlcNAc Pento	5-Me-β-L-Rha	Pn23

Figure 2. Photogenerated glycan arrays recognize immunogenic sugar moieties of the *B. anthracis* spore. A panel of 35 mono-, oligo-, and polysaccharides (see Supplementary Table 1 for structural information) were photocoupled on glass slides. After incubating the glycan arrays with rabbit anti-*B. anthracis* spore polyclonal IgG antibodies (Abcam, Cambridge, UK; the same antibody preparation was used in the experiments of Fig. 3) in the absence or presence of saccharide inhibitors, the bound rabbit IgG was revealed by a tagged antirabbit IgG antibody. Glycan array staining, image capturing, and data-processing were performed as previously described [15, 19–22]. Microarray datasets are shown in Supplementary Table 2.

Figure 3a illustrates a quantitative comparison of the relative IgG antibody reactivities detected by the glycan arrays. Each histogram displays the mean intensity of triplicate detections by the glycan arrays. The antibody reactivity with anthrose-containing saccharides correlates positively with the sizes of the saccharides immobilized on the glycan arrays. The anthrose monosaccharide (Saccharide ID #1) and anthrose-containing disaccharide (ID #2) are marginally reactive while the anthrose-containing tetrasaccharides (ID #4 and #5) are highly reactive, regardless of their configuration at the anomeric center carrying the aglycone. Such antibody reactivities were not detected in a collection of four preimmunized rabbit sera and 12 rabbit antisera against irrelevant antigens. These results demonstrate that rabbit IgG antibodies elicited by the native spore antigens recognize the synthetic carbohydrate moieties of the BclA glycoprotein.

In order to grade the immunodominance of sugar moieties of the *B. anthracis* exosporium, we conducted microarray-based saccharide blocking assays (Fig. 3b). The microarray assay simultaneously measures the relative antigenic reactivities of antibodies with multiple antigenic structures. Introducing a specific saccharide as a competitor into this multiplex antigen–antibody interaction provides a critical measurement of the specificity and crossreactivity of a specific antibody preparation. If an antibody fingerprint, *i.e.* an array of positively stained microspots of a given saccharide structure, would be blocked by a specific saccharide, we could infer that the specificity of the reacting antibody is specific for the corresponding saccharide structure. Inhibition of a number of different antibody fingerprints by a given saccharide would suggest that these reactive saccharide structures share common or crossreactive sugar epitopes.

We scanned a panel of monosaccharides and glycosides to identify potential inhibitors, including 5-methoxycarbonyl β-anthroside, L-rhamnose, and other common sugar residues listed in the Supplementary Table 1. Results summarized in Fig. 3b show that the anthrose glycoside blocked the antibody reactivity with anthrose-containing tri- and tetrasaccharides (α- and β-glycosides) (Figs. 2 and 3). In contrast, the same anthrose glycoside shows no significant inhibition of other antibody reactivities observed with the same rabbit polyclonal antibodies, such as binding to the polysaccharide isolichenin [α(1,3)-glucan] (Fig. 2, positive spots in the upper-left corner) and a preparation of Pn 23 polysaccharide (Fig. 2, positive spots in the bottom-right corner). We also examined anthrose-containing di, tri, and tetrasaccharides and observed that their inhibition profiles or specificities are identical to those mediated by the anthrose monosaccharide, *i.e.* they competitively inhibit the specific antibody reactivities with the anthrose-containing saccharide moieties but not other anticarbohydrate reactivities present in the polyclonal rabbit IgG preparation (Figure 3b).

In order to determine the relative contribution of sugar residues of the anthrose-containing tetrasaccharide to the antibody binding, we performed ELISA-based quantitative

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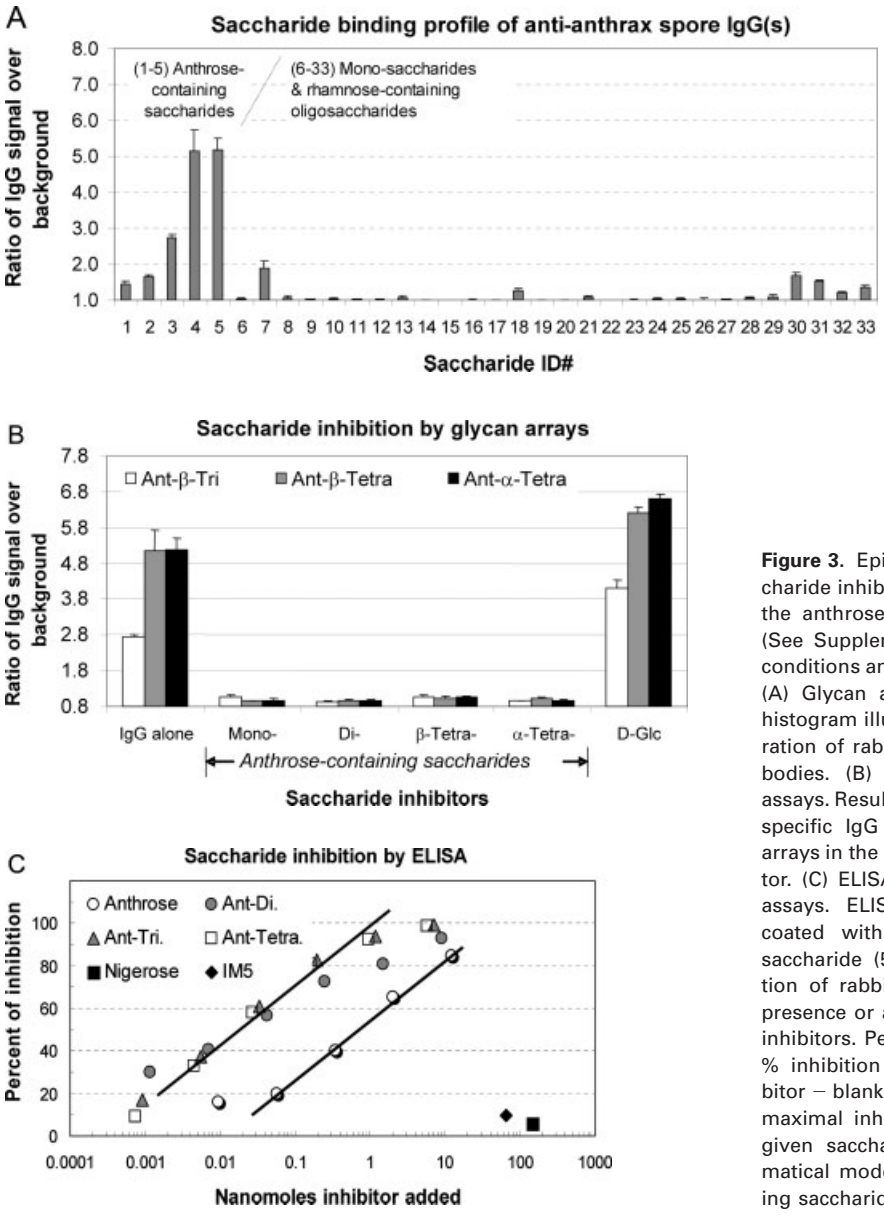

Figure 3. Epitope-mapping using glycan array and saccharide inhibition assays to identify the key elements of the anthrose-containing immunogenic sugar moieties (See Supplementary Tables 2 and 3 for glycan assay conditions and microarray datasets).
(A) Glycan array-based saccharide-binding assays: A histogram illustration of the antibody profile of a preparation of rabbit anti-*B. anthracis* spore polyclonal antibodies. (B) Glycan array-based saccharide blocking assays. Results are illustrated as the levels of saccharide-specific IgG antibodies detected by triplicate glycan arrays in the presence or absence of a saccharide inhibitor. (C) ELISA-based quantitative saccharide inhibition assays. ELISA microtiter plates (NUNC, MaxiSorp) coated with a BSA conjugate of α-anthrose-tetrasaccharide (5 μg/ml) were incubated with a preparation of rabbit antianthrax spore IgG (2 μg/mL) in the presence or absence of varying quantities of the sugar inhibitors. Percent inhibition was calculated as follows: % inhibition = ((standard A − blank A) − (A with inhibitor − blank A))/(standard A − blank A). The half maximal inhibitory concentration (IC_{50}) values for the given saccharides were calculated based on mathematical models of the linear range of the corresponding saccharide inhibition curve [23].

saccharide inhibition assays using anthrose and anthrose-containing di-, tri-, and tetrasaccharides. In this assay, an anthrose-tetrasaccharide-BSA conjugate was coated on an ELISA plate at 5 μg/ml in 0.1M sodium bicarbonate buffer, pH 9.6 and the corresponding saccharides were mixed with antianthrax spore IgG antibodies in the binding reaction. As shown in Fig. 3c, the quantitative inhibition curves generated by the four saccharides are nearly linear. The curve of the anthrose-trisaccharide (IC_{50}, 0.016 nmol) is essentially superimposed to the curve of the anthrose-tetrasaccharide (IC_{50}, 0.016 nmol). Both marginally differ from the disaccharide (IC_{50}, 0.019 nmol) but are significantly different from the monosaccharide (IC_{50}, 0.688 nmol). The relative inhibiting powers of the four anthrose saccharides are, thus, 1.00(tetra)/1.00(tri)/1.18(di)/43.3(mono) [23]. In striking contrast, an α(1,3)glucosyl disaccharide, nigerose, and an α(1,6)glucosyl pentasaccharide, IM5 (isomaltopentose), show no inhibition to the specific antibody reactivities with the anthrose-tetrasaccharide in the same assay.

Taking together, we demonstrate that IgG antibodies elicited by the native antigens of the *B. anthracis* spore recognize synthetic anthrose-containing sugar moieties. The saccharide-binding reactivities correlate directly with the sizes of the saccharides displayed by the glycan arrays. The terminal anthrose monosaccharide is marginally reactive and the anthrose-containing tetrasaccharides highly reactive, regardless of their anomeric configuration. However, the smaller saccharide units, including the anthrose mono-, di-, and tri-

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saccharides are potent inhibitors of the specific antibody reactivities to the tetrasaccharides displayed either by the photogenerated glycan arrays or by a BSA conjugate on an ELISA microtiter plate. We conclude, therefore, that the anthrose-containing tetrasaccharide is immunogenic in its native configuration as displayed by the exosporium BclA glycoprotein and its terminal trisaccharide unit is essential for the constitution of an highly specific antigenic determinant.

Given the fact that this carbohydrate moiety is displayed on the outermost surfaces of *B. anthracis* spores [11, 12] and its expression is highly specific for the spore of *B. anthracis* [12], the anthrose-containing tetrasaccharide can be considered an important immunological target. Its applications may include identification of the presence of *B. anthracis* spores, surveillance and diagnosis of anthrax infection, and development of novel vaccines targeting the *B. anthracis* spore. Effort must also be made to explore the biological role of this highly specific carbohydrate moiety of *B. anthracis*. Generally, the experimental approach we demonstrated here allows high-throughput screening of libraries of saccharide structures for their potential antigenic reactivities. Its application may substantially facilitate the identification of key immunogenic sugar moieties of microbial pathogens.

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