DOI: 10.1002/cbic.200800210

Chemical Synthesis and Immunological Properties of Oligosaccharides Derived from the Vegetative Cell Wall of *Bacillus anthracis*

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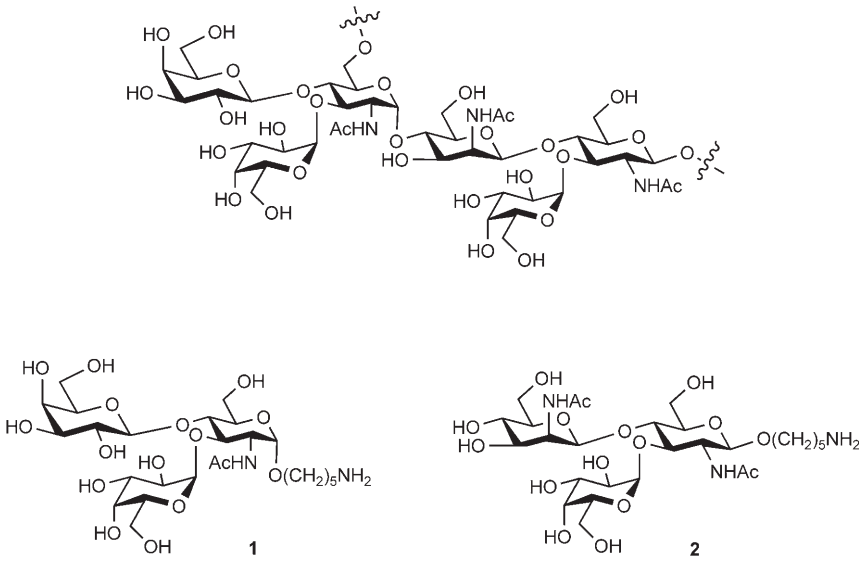
Bacillus anthracis is a Gram-positive, spore-forming bacterium that causes anthrax in humans and other mammals.[1,2] The relative ease by which *B. anthracis* can be weaponized and the difficulty associated with the early recognition of inhalation anthrax due to the unspecific nature of its symptoms were underscored by the deaths of five people who inhaled spores from contaminated mail.[3–5] As a result, there is a renewed interest in anthrax vaccines and early-disease diagnostics.[6]

Anthrax vaccine adsorbed (AVA; BioThrax®, Emergent BioSolutions, Inc.) is currently the only licensed anthrax vaccine in the US.[7,8] The principal immunogen of AVA is anthrax toxin protective antigen (PA). Antibody responses against PA target and block the toxemia that is a necessary prerequisite of vegetative cell growth and bacteremia. Vaccines comprising additional *B. anthracis* specific antigens have been proposed as improvements to PA-only formulations as they have the potential to target inclusively the toxemia and the vegetative cell or infectious spore.[9–11] Recently described polysaccharides and glycoproteins of *B. anthracis* offer exciting new targets for these vaccine formulations and also for the development of improved diagnostics for *B. anthracis*. For example, an unusual oligosaccharide derived from the collagen-like glycoprotein Bc1A of the exosporium of *B. anthracis* has been characterized,[12] chemically synthesized,[13–18] and immunologically evaluated. The latter studies demonstrated that the oligosaccharide is exposed to the immune system[14] and has the ability to elicit relevant antibodies.[13]

Recently, we reported the structure of a unique polysaccharide released from the vegetative cell wall of *B. anthracis*, which contains a →6)-α-D-GlcNAc-(1→4)-β-D-ManNAc-(1→4)-β-D-GlcNAc-(1→) backbone and is branched at C-3 and C-4 of α-D-GlcNAc with α-D-Gal and β-D-Gal residues, respectively, and the β-GlcNAc substituted with α-Gal at C-3 (Scheme 1).[19,20] These positions are, however, only partially substituted and this leads to microheterogeneity.



Scheme 1. Structure of the secondary cell wall polysaccharide of *B. anthracis* and synthetic compounds **1** and **2**.

As part of a project to determine antigenic determinates of the polysaccharide of *B. anthracis* and to establish it as a diagnostic or vaccine candidate, we report here the chemical synthesis and immunological properties of trisaccharides **1** and **2** (Scheme 1). These compounds, which are derived from *B. anthracis* polysaccharide, contain a 5-aminopentyl spacer for selective conjugation to carrier proteins required for enzyme-linked immunosorbent assays (ELISA). It has been found that sera from rabbits either exposed to live and irradiation-killed spores of *B. anthracis* Sterne $34F_2$ or immunized with polysaccharide conjugated to keyhole limpet hemocyanin (KLH) recognize the isolated polysaccharide and synthetic compounds **1** and **2**. The data provide proof-of-concept for the development of vegetative and spore-specific reagents for detection and targeting of nonprotein structures of *B. anthracis*.

Compound **1** was conveniently prepared from monosaccharide building blocks **3**,[21] **4**, and **7**.[22] Thus, a NIS/TMSOTf medi-

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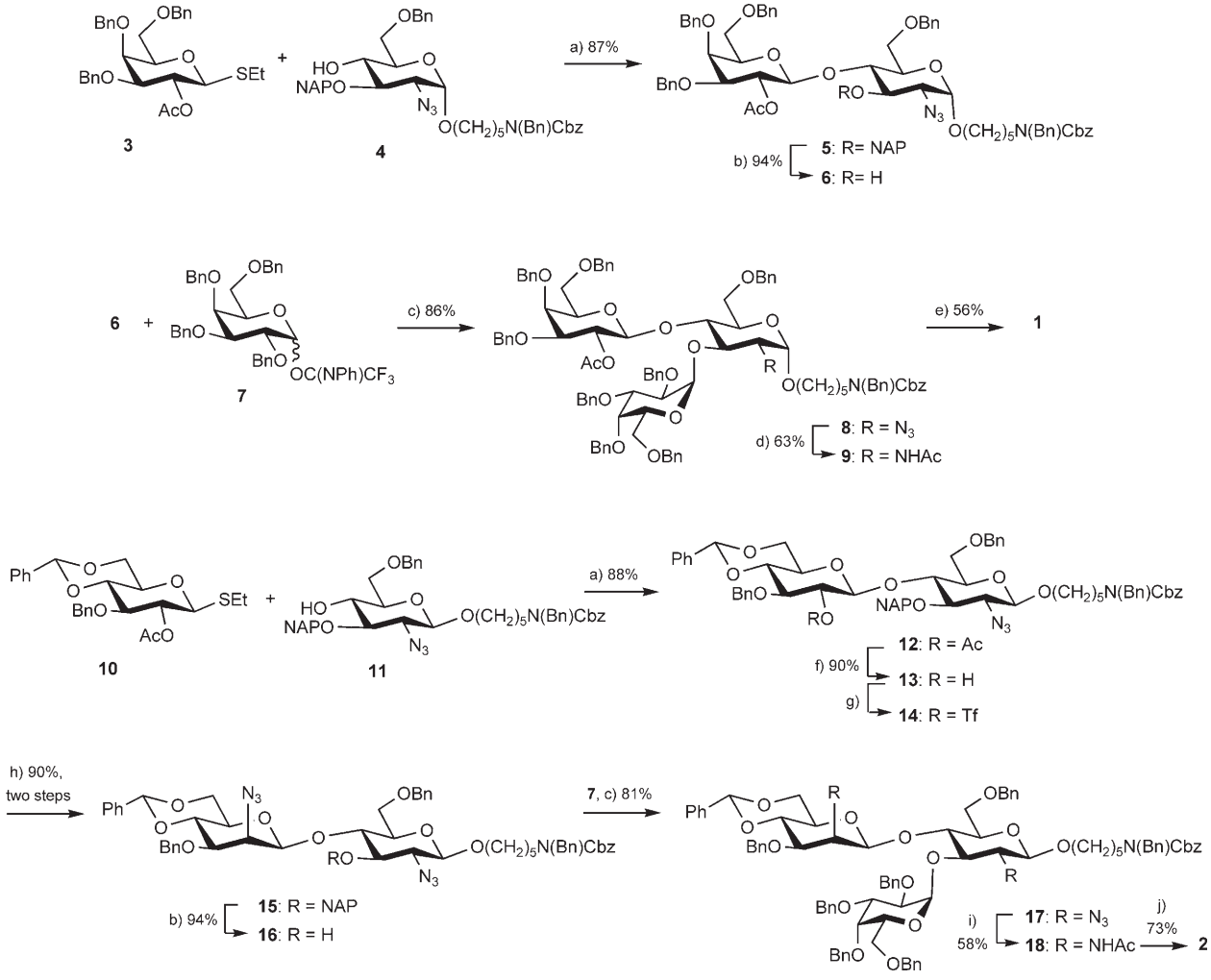
ated glycosylation[23] of thioglycoside **3** with the C-4 hydroxyl of glycosyl acceptor **4** gave disaccharide **5** in a yield of 87% as only the β-anomer (Scheme 2). Interestingly, a lower yield of disaccharide was obtained when a glycosyl acceptor was employed that had a benzyloxycarbonyl-3-aminopropyl instead of a *N*-benzyl-*N*-benzyloxycarbonyl-5-aminopropyl spacer.[24] Next, the 2-naphthylmethyl ether[25,26] of **5** was removed by oxidation with DDQ in a mixture of dichloromethane and water to give glycosyl acceptor **6**, which was used in a TMSOTf mediated glycosylation with (*N*-phenyl)trifluoracetimidate (**7**)[27–29] to afford trisaccharide **8** in excellent yield as only the α-anomer. The use of a conventional trichloroacetimidate as glycosyl donor[30] led to a lower yield of product due to partial rearrangement to the corresponding anomeric amide. Target compound **1** was obtained by a three-step deprotection procedure that involved reduction of the azide to an acetamido moiety by treatment with $Zn/CuSO_4$[31] in a mixture of acetic anhydride, acetic acid, and THF, followed by saponification of the acetyl ester and reductive removal of benzyl ethers and benzyloxycarbamate by catalytic hydrogenation over Pd.

A challenging aspect of the preparation of target compound **2** is the installment of a β-mannosamine moiety.[27] A strategy was adopted in which a β-glucoside is initially installed by using a glucosyl donor that has a participating ester-protecting group at C-2 to control β-anomeric selectivity.[32] Next, the C-2 protecting group can be removed and the resulting hydroxyl triflated, which can then be displaced by an azide to give a 2-azido-β-D-mannoside. Another strategic aspect of the synthesis of **2** was the use of an acetyl ester and 2-naphtylmethyl ether[25,26] as a set of orthogonal-protecting groups, which makes it possible to selectively modify C-2′ of the β-glucoside and install an α-galactoside at C-3 of 2-azido-glucoside moiety. Thus, a NIS/TMSOTf mediated glycosylation[23] of thioglycoside **10**[33] with **11** gave disaccharide **12** in excellent yield as only the β-anomer. The acetyl ester of **12** was saponified by treatment with sodium methoxide in methanol to give **13**. Next, the alcohol of **13** was triflated by treatment with triflic anhydride in a mixture of pyridine and dichloromethane to afford triflate **14**, which was immediately displaced with sodium azide in DMF at 50 °C to give mannoside **15**. The 2-naphthyl-



Scheme 2. Reagents and conditions. a) NIS/TMSOTf, DCM, 0 °C; b) DDQ, DCM, H_2O; c) TMSOTf, DCM, Et_2O, 50 °C; d) $Zn/CuSO_4$, AcOH, Ac_2O, THF; e) NaOMe, MeOH then $Pd(OH)_2/C$, H_2, AcOH, *t*BuOH, H_2O; f) NaOMe, MeOH; g) Tf_2O, pyridine, DCM, 0 °C; h) NaN_3, DMF, 50 °C; i) PMe_3, THF, H_2O then Ac_2O, pyridine; j) $Pd(OH)_2/C$, H_2, AcOH, *t*BuOH, H_2O.

methyl ether of **15** was removed by oxidation with DDQ,[26] and the resulting glycosyl acceptor **16** was glycosylated with **7** in the presence of a catalytic amount of TMSOTf in a mixture of dichloromethane and diethyl ether to give anomerically pure trisaccharide **17**. Deprotection of **17** was accomplished by reduction of the azides with trimethyl phosphine[34] followed by acetylation of the resulting amine with acetic anhydride in pyridine, and then reductive removal of the benzyl ethers and benzyloxycarbamate by catalytic hydrogenation over Pd to give compound **2**.

For immunological evaluations, trisaccharides **1** and **2** were conjugated to bovine serum albumin (BSA) by treatment with *S*-acetylthioglycolic acid pentafluorophenyl ester to afford the corresponding thioacetate derivatives, which after purification by size-exclusion chromatography were de-*S*-acetylated by using 7% ammonia (g) in DMF and conjugated to maleimide activated BSA (BSA–MI, Pierce Endogen, Inc.) in phosphate buffer (pH 7.2). After purification by using a centrifugal filter device with a nominal molecular weight cut-off of 10 kDa, neoglycoproteins were obtained with an average of eleven and nineteen molecules of **1** and **2**, respectively, per BSA molecule as determined by Bradford's protein assay and quantitative carbohydrate analysis by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

Next, conjugates of KLH and BSA to the polysaccharide of *B. anthracis* were prepared for immunization of rabbits and examination of antisera for anti-polysaccharide antibodies, respectively. To this end, the polysaccharide was treated with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP)[35] to form reactive cyanyl esters, which were condensed with free amines of BSA and KLH to give, after rearrangement of isourea-type intermediate, carbamate-linked polysaccharides. The KLH– and BSA–polysaccharide conjugate solutions were purified by using centrifugal filter devices (Micron YM 30000 Da) and then lyophilized. Saccharide loadings of 0.3 mg per mg of BSA and 0.96 mg per mg of KLH were determined by using bicinchoninic acid (BCA; BSA conjugate) and Bradford's (KLH conjugate) protein assays and quantitative carbohydrate analysis by HPAEC-PAD. In addition, maltoheptaose was conjugated to BSA by using CDAP to obtain a control conjugate to examine for the possible presence of anti-linker antibodies.[36]

Rabbits were inoculated intramuscularly four times at biweekly intervals with live- or irradiated spores (3×10^6 total spores),[14] or polysaccharide–KLH conjugate, followed by the collection of terminal bleeds fourteen days after the last immunization. ELISA was used to examine the pre- and postimmune sera for polysaccharide recognition. Microtiter plates were coated with the polysaccharide–BSA conjugate and serial dilutions of sera added. An anti-rabbit IgG antibody labeled with horseradish peroxidase was employed as a secondary antibody for detection purposes. High titers of anti-polysaccharide IgG antibodies had been elicited by the polysaccharide–KLH conjugate (Figure 1 A, Table 1). Furthermore, inoculation with live

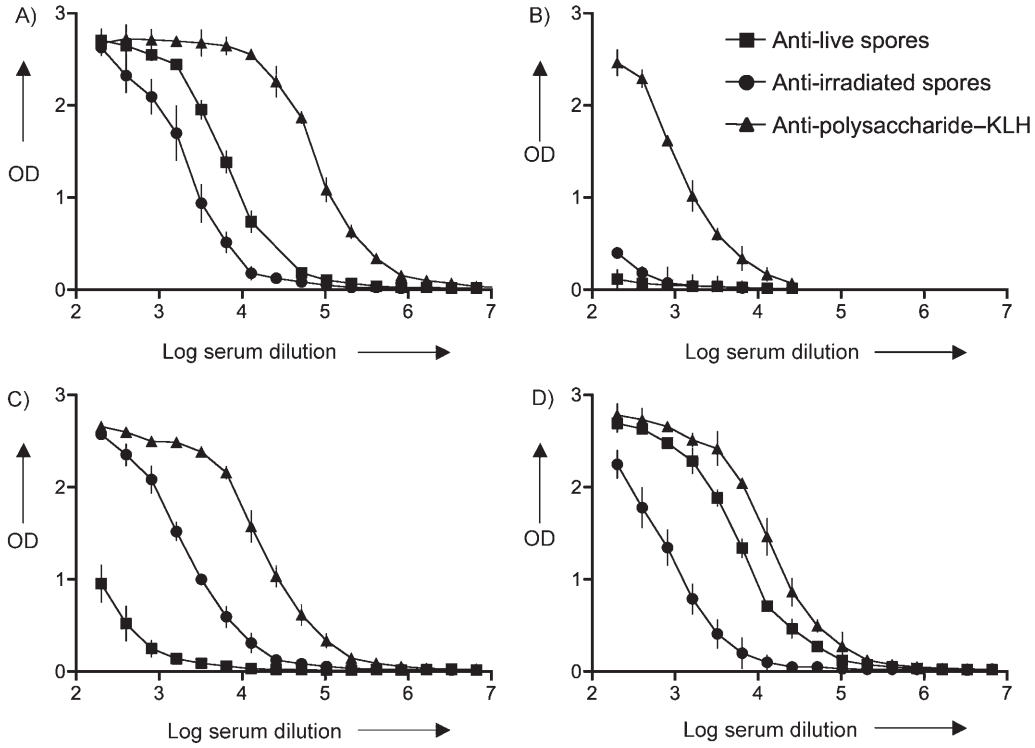


Figure 1. Immunoreactivity of polysaccharide and trisaccharides **1** and **2** to antisera elicited by *B. anthracis* Sterne live spores, irradiation-killed spores, and polysaccharide–KLH conjugate. Microtiter plates were coated with A) polysaccharide–BSA, B) maltoheptaose–BSA, C) **1**–BSA, and D) **2**–BSA conjugates (0.15 $\mu g\,mL^{-1}$ carbohydrate). Serial dilutions of rabbit anti-live and anti-irradiated *B. anthracis* Sterne 34F2 spores antisera and rabbit anti-polysaccharide–KLH antiserum (starting dilution 1:200) were applied to coated microtiter plates. Serial dilutions of the preimmune sera from the rabbits (starting dilution 1:200) did not show any binding to polysaccharide–BSA (data not shown). Wells only coated with BSA at the corresponding protein concentration did not show binding to any sera (data not shown). The optical density (OD) values are reported as the means ± SD of triplicate measurements.

Table 1. ELISA antibody titers after immunization with *B. anthracis* Sterne live spores, irradiation-killed spores, and polysaccharide–KLH.

Immunization coating	Live spores	Irradiated spores	Polysaccharide–KLH
polysaccharide–BSA	18500	6100	239700
maltoheptaose–BSA	0	0	3600
1–BSA	400	6800	57300
2–BSA	18700	2600	46700

ELISA plates were coated with BSA conjugates (0.15 μg mL^{-1} carbohydrate) and titers were determined by linear regression analysis by plotting dilution versus absorbance. Titers are defined as the highest dilution that yielded an optical density of 0.5 or greater.

and irradiated spores resulted in the production of IgG antibodies that could recognize the polysaccharide. Antisera obtained from immunizations with polysaccharide–KLH conjugate showed recognition of maltoheptaose linked to BSA albeit at much lower titers than when polysaccharide–BSA was used as ELISA coating. This finding indicates that some anti-linker antibodies had been elicited.[36] As expected, antisera from rabbits immunized with live and irradiated spores showed no reactivity towards the maltoheptaose conjugate (Figure 1B).

Next, the specificity of the anti-polysaccharide antibodies was investigated by using synthetic trisaccharides **1** and **2** (Scheme 1) linked to BSA. Trisaccharides **1** and **2** were equally well recognized by IgG antibodies that were elicited by the polysaccharide–KLH conjugate and irradiation-killed spores (Figure 1C and D, Table 1). Surprisingly, antisera obtained after inoculation with live spores recognized trisaccharide **2** much better than **1**.

To further study the antigenic components of the various antisera, inhibition ELISAs were performed by coating microtiters plates with polysaccharide–BSA conjugate and by using **1**–BSA, **2**–BSA, and polysaccharide–BSA as inhibitors (Figure 2). As expected, for each antiserum, the polysaccharide–BSA inhibitor could completely block the binding of IgG antibodies to immobilized polysaccharide, whereas only partial inhibition was observed for **1**–BSA and **2**–BSA. Furthermore, antibodies elicited by the live spore vaccine recognized trisaccharide **2** much better than **1**, whereas the polysaccharide–KLH antiserum was better inhibited by **1**. Antibodies elicited by the irradiated spore inoculum recognized **1** and **2** equally well. The partial inhibition by the synthetic compounds indicates that heterogeneous populations of antibodies were elicited. Furthermore, the difference in antigenic component of the vaccines might be due to differences in presentation of the polysaccharide when it is part of vegetative cells, or attached to KLH, or when it is part of irradiation-killed spores.

The results presented here show that both live- and irradiation-killed *B. anthracis* spore inoculae and polysaccharide linked to the carrier protein KLH can elicit IgG antibodies that recognize isolated polysaccharide and the relatively small saccharides **1** and **2**. Previously, the polysaccharide was identified

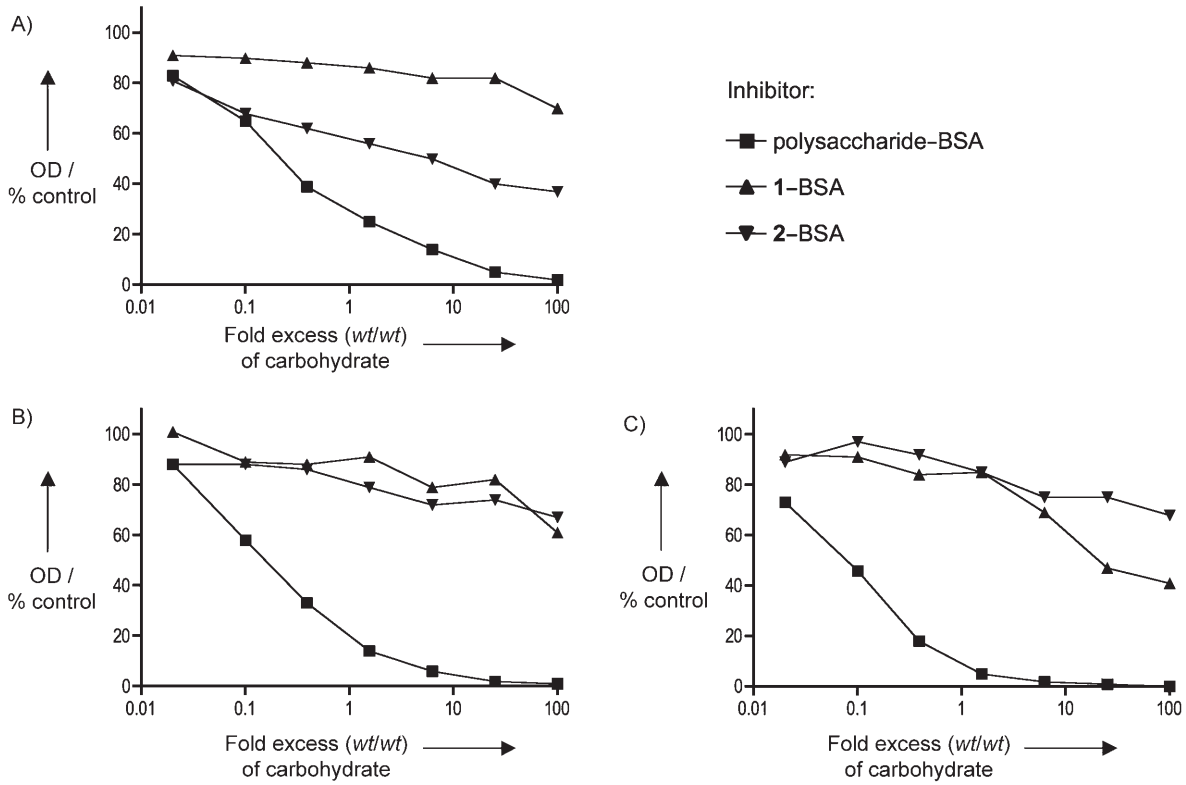


Figure 2. Competitive inhibition ELISA. Microtiter plates were coated with polysaccharide–BSA conjugate (0.15 μg mL^{-1} carbohydrate). Dilutions of A) rabbit anti-live, B) anti-irradiated *B. anthracis* Sterne 34F2 spores antisera, and C) rabbit anti-polysaccharide–KLH antiserum mixed with polysaccharide–BSA, **1**–BSA, and **2**–BSA (0–100-fold excess, *wt*/*wt*, based on carbohydrate concentration) were applied to coated microtiter plates. Maltoheptaose–BSA conjugate and unconjugated BSA at corresponding concentrations mixed with antisera did not display inhibition (data not shown). OD values were normalized for the OD values obtained in the absence of inhibitor (0-fold "excess", 100%).

as a component of the vegetative cell wall of *B. anthracis*, and thus, it was surprising that irradiation-killed spores could elicit anti-polysaccharide antibodies. It appears that not only vegetative cells but also *B. anthracis* spores express the polysaccharide. The implication of this finding is that a polysaccharide-based vaccine could provide immunity towards vegetative cells as well as spores. In this respect, we hypothesize that immune responses to dormant *B. anthracis* spores at the mucosal surface might inhibit spore uptake across the mucosa and might also target the susceptible emergent vegetative cell; this would either prevent bacterial proliferation or enhance bacterial clearance. Highly conserved integral carbohydrate components of the spore and vegetative cell structure are attractive vaccine candidate antigens because unlike capsules, they are not sloughed off the replicating cell. Finally, we have located important antigenic components of the various antisera using synthetic saccharides.

The data provide an important proof-of-concept step in the development of vegetative and spore-specific reagents for detection and targeting of nonprotein structures in *B. anthracis*. These structures might in turn provide a platform for directing immune responses to spore structures during the early stages of the *B. anthracis* infection process. Ongoing studies will demonstrate whether anti-polysaccharide antibodies can recognize *B. anthracis* spores, including the highly virulent *B. anthracis* Ames and *B. anthracis* cured of virulence plasmids (pXO1 and pXO2). Examination of the cross reactivity of the antisera with cell wall polysaccharides from various *Bacillus* species and determination of antigenic responses against the synthetic oligosaccharides are also underway.[37]

Acknowledgements

This research was supported by the Institute of General Medicine of the National Institutes of Health, grant GM065248 (G.J.B.) and NIAID, grant AI059577 (R.W.C.). We wish to thank Dr. Therese Buskas for the preparation of the BSA conjugates and Dr. Elke Saile for assistance with rabbit immunizations. The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the Centers for Disease Control and Prevention.

Keywords: *Bacillus anthracis* · glycoconjugates · oligosaccharides · protein conjugation · vaccines

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Received: April 1, 2008
Published online on June 18, 2008

