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Article

A Possible Oligosaccharide-Conjugate Vaccine Candidate for *Clostridium difficile* Is Antigenic and Immunogenic

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SUMMARY

Nosocomial infections with the Gram-positive pathogen *Clostridium difficile* pose a major risk for hospitalized patients and result in significant costs to health care systems. Here, we present the chemical synthesis of a PS-II hapten of a cell wall polysaccharide of hypervirulent ribotype 027 of *C. difficile*. Mice were immunized with a conjugate consisting of the synthetic hexasaccharide and the diphtheria toxoid variant CRM_{197}. The immunogenicity of the glycan repeating unit was demonstrated by the presence of specific IgG antibodies in the serum of immunized mice. Murine monoclonal antibodies interact with the synthetic hexasaccharide, as determined by microarray analysis. Finally, we found that specific IgA antibodies in the stool of hospital patients infected with *C. difficile* recognize the synthetic PS-II hexasaccharide hapten.

INTRODUCTION

The Gram-positive bacteria of the genus *Clostridium difficile* have long been recognized as the cause of a range of gastrointestinal diseases (Hookman and Barkin, 2009). Infection and the development of *C. difficile*-associated diseases (CDADs) are linked to the use of antibiotics that disrupt the normal intestinal flora and allow for proliferation of *C. difficile* (Thomas et al., 2003). *C. difficile* infection in its most severe form can cause toxic megacolon with subsequent colonic perforation, peritonitis, shock, and death. Furthermore, *C. difficile* is a major cause of diarrhea in hospital and long-term care facility patients due to the frequent use of antibiotics, contamination of these facilities with resistant spores, and because of the high density of susceptible persons. A dramatic increase in *C. difficile* incidents was recorded in many developed countries (Polk et al., 2006) starting with reports of hospital outbreaks in Canada in 2003 (Pépin et al., 2004). With the increasing severity of the incidents, relapse and mortality rates also increased significantly (Kuijper et al., 2007). The North American and European outbreaks coincided with the emergence of a hypervirulent strain of *C. difficile*, alternatively designated by the synonymous terms as PCR ribotype 027, toxin type III, NAP1, and BI (McDonald et al., 2005; Loo et al., 2005). The hypervirulence of ribotype 027 has been ascribed to its higher toxin yields and an increased rate of sporulation (Åkerlund et al., 2008). Higher toxin content is due to an additional toxin referred to as the binary toxin and a genetic mutation in a toxin regulator gene (*tcdC*), encoding a negative regulator of the *C. difficile* pathogenicity locus. The isolates obtained during the North American and European epidemics were genetically closely related and, in addition, resistant to fluoroquinolones (Clements et al., 2010).

The medical significance of *C. difficile* has prompted intense studies aiming to elucidate the structural composition of its cell wall. Two capsular polysaccharides, PS-I and PS-II, were identified (Ganeshapillai et al., 2008). PS-I has a branched pentaglycosyl phosphate repeating unit, and PS-II a hexaglycosyl phosphate repeating unit. Both are found on the highly virulent ribotype 027 (Figure 1). Currently, to our knowledge, no licensed vaccine against *C. difficile* is available.

Several marketed vaccines are based either on natural polysaccharides alone (Lucas and Reason, 1999) or on polysaccharides linked to immunogenic protein carriers (Hecht et al., 2009; Roy, 2008). Polysaccharide-protein conjugate vaccines based on isolated carbohydrates are an effective measure against at least five different bacteria: *Haemophilus influenzae* type b, *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Salmonella typhi*, and *Staphylococcus aureus* infections (Ada and Isaacs, 2003). A synthetic carbohydrate-protein conjugate vaccine against *Haemophilus influenzae* type b was developed and marketed in Cuba (Verez-Bencomo et al., 2004; Roy 2008).

Here, we report the synthesis of a hapten of the *C. difficile* PS-II, its conjugation to the diphtheria toxoid CRM_{197}, and the production of monoclonal antibodies that specifically recognize the glycan hapten. Polysaccharide-specific IgA antibodies were detected in patients diagnosed with *C. difficile* infections. The PS-II structure was selected as synthetic target because it was reported to be found on several *C. difficile* strains, including ribotype 027 (Ganeshapillai et al., 2008).

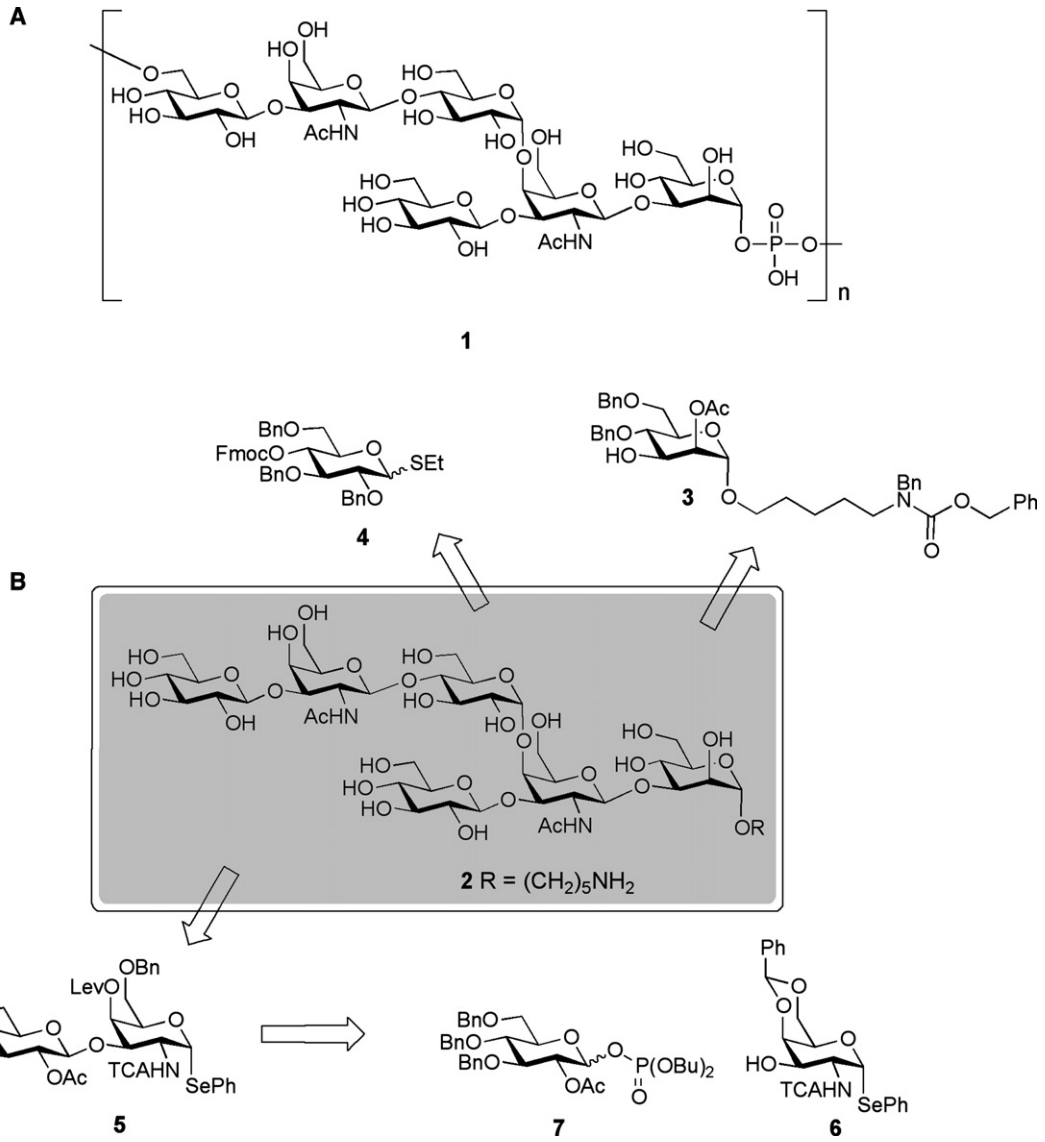

Figure 1. Retrosynthetic Analysis of Hexasaccharide Repeating Unit 2
(A) Structure of PS-II found on *C. difficile*.
(B) Retrosynthetic analysis of PS-II hapten analog **2**.

RESULTS

Oligosaccharide Repeating Unit Synthesis

The PS-II repeating units are interconnected via a (1→6) phosphate diester linkage in the natural polysaccharide (Figure 1). We aimed to synthesize oligosaccharide **2** (Figure 1), which constitutes a nonphosphorylated PS-II hexasaccharide hapten. The oligosaccharide was designed to carry a primary amine at the reducing terminus via a spacer to facilitate conjugation to a protein carrier and attachment to microarrays. Based on our retrosynthetic analysis, the hexasaccharide will be assembled from the monosaccharide building blocks **3** and **4**, and the disaccharide building block **5** that appears twice in the target structure. Disaccharide **5** will be derived in turn from monosaccharide building blocks **6** and **7**. The building blocks **3**, **4**, **6**, and **7** were synthesized from commercially available unprotected monosaccharides following known methodology.

Preparation of the reducing terminus commenced with glycosylation of the protected spacer *N*-benzyl-*N*-benzyloxycarbonyl-5-aminopentanol **8** (Delcros et al., 2002) with mannose building block **9** (Figures 1 and 2). The 2-*O*-benzoyl and 3-*O*-levulinoyl protection groups on building block **9** were crucial for the success of this glycosylation because when the alternative mannose-based building block bearing 2-*O*-acetate and 3-*O*-fluorenylmethyloxycarbonyl (Fmoc) protecting groups was used instead, it afforded mainly the orthoester product (Kong, 2007; Ravidà et al., 2006) and only small amounts of the desired product. The levulinoyl ester in mannose glycoside **10** was selectively cleaved using hydrazine monohydrate to reveal the C3 hydroxyl group to give glycosyl acceptor **11**.

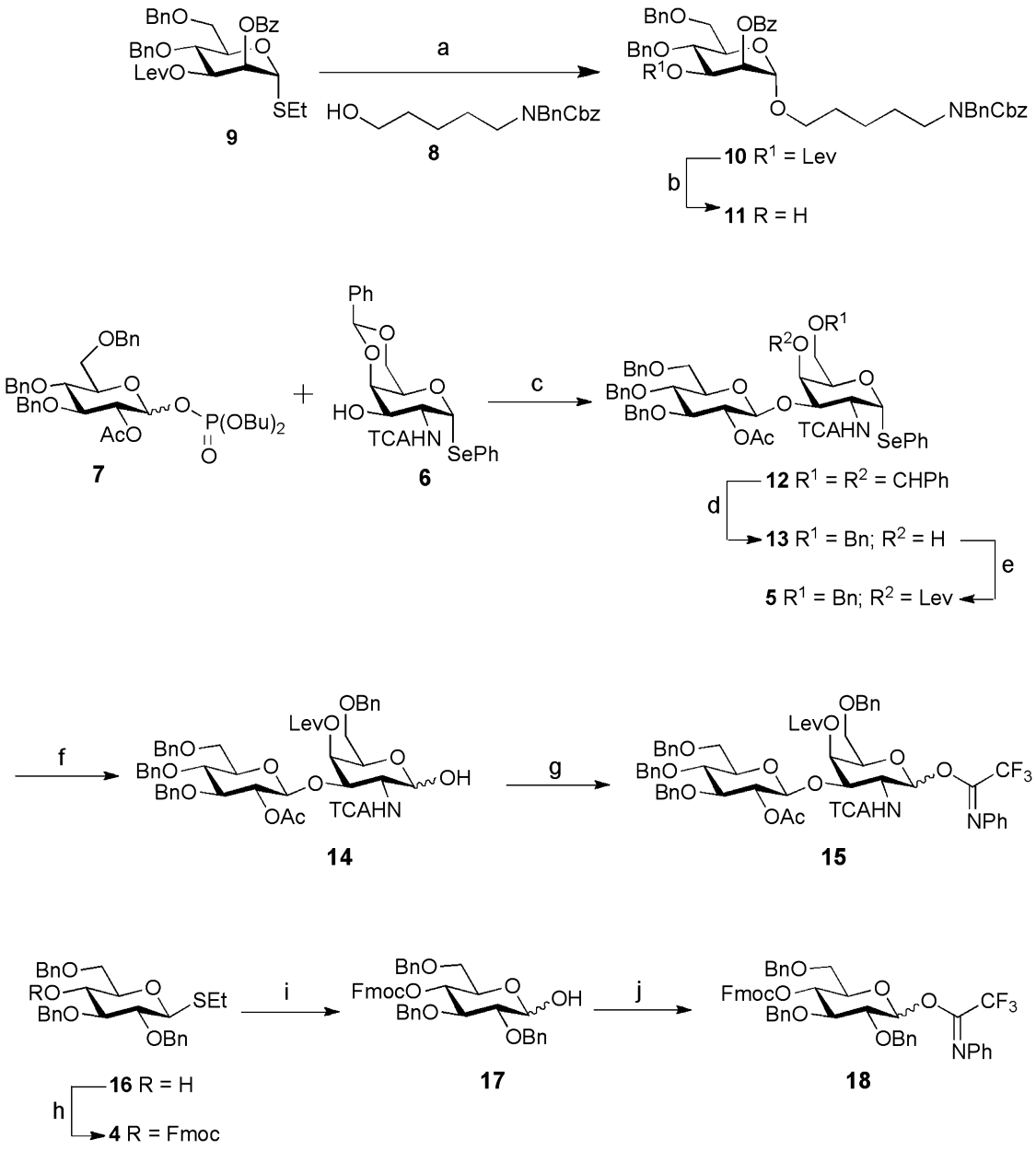

Figure 2. Synthesis of Building Blocks 11, 15, and 18

Reagents and conditions: (a) NIS, TMSOTf, CH_2Cl_2, 90%; (b) $N_2H_4 \cdot H_2O$, AcOH, Py, quantitative; (c) TMSOTf, CH_2Cl_2, −30°C, 78%; (d) Et_3SiH, TfOH, CH_2Cl_2, −78°C, 68%; (e) LevOH, DMAP, DIPC, CH_2Cl_2, 94%; (f) NIS, aqueous HCl, THF, 96%; (g) $CF_3C(NPh)Cl$, Cs_2CO_3, CH_2Cl_2, 78%; (h) FmocCl, Py, CH_2Cl_2, 72%; (i) NBS, aqueous HCl, THF, 70%; (j) $CF_3C(NPh)Cl$, Cs_2CO_3, CH_2Cl_2, quantitative. Lev, Levulinoyl; Bn, benzyl; Bz, benzoyl; Cbz, benzyloxycarbonyl; TCA, trichloroacetyl.

Disaccharide building block **5** resulted from the union of galactosamine **6** (Chen et al., 2005) with the previously characterized glucosyl phosphate **7** (Ravidà et al., 2006) via trimethylsilyl trifluoromethanesulfonate-mediated activation of the anomeric dibutyl phosphate leaving group in **7** followed by nucleophilic substitution by **6** to afford disaccharide **12** (Figures 1 and 2). The selective opening of the benzylidene acetal in compound **12**, to afford disaccharide **13** bearing a 6-*O*-benzyl protection group and no protection group on the C4 hydroxyl group, strongly depended on the reaction conditions. Best results were obtained when disaccharide **12** was treated with triethylsilane and triflic acid at −78°C. Other methods that relied on using trimethylsilyl trifluoromethanesulfonate as Lewis acid, or sodium cyanoborohydride as a reducing agent, furnished inseparable mixtures of the 4-hydroxyl and 6-hydroxyl-regioisomers. The free C4 hydroxyl group in disaccharide **13** was subsequently masked as levulinoyl ester to afford glycosylating agent **5**.

Hexasaccharide assembly commenced with the glycosylation of monosaccharide **11** with phenyl selenide **5** to furnish trisaccharide **19** with a yield of up to 61% (Depré et al., 1999). To

improve the coupling yields, we changed the synthetic strategy by replacing the anomeric leaving group in **5**. The phenyl selenide in **5** was replaced by a different anomeric leaving group, by a *N*-phenyl trifluoroacetimidate group (Yu and Tao, 2001). Disaccharide **5** was converted, via lactol **14**, to the corresponding glycosyl *N*-phenyl trifluoroacetimidate **15**. As envisioned, glycosylation of nucleophile **11** with disaccharide *N*-phenyl trifluoroacetimidate **15** yielded 82% of the desired product, which compared favorably to the 61% obtained when phenyl selenide **5** was used as disaccharide-glycosylating agent. The C2 participating trichloroacetamido group of galactosamine in **15** ensured the exclusive formation of the β linkage. Treatment of trisaccharide **19** with hydrazine monohydrate resulted in cleavage of the levulinoyl ester and furnished **20** bearing a free hydroxyl group ready for the next glycosylation. Glycosylation of trisaccharide **20** with thioglycoside **4** afforded a 55% yield of tetrasaccharide **21**. The yield of this glycosylation was again improved when *N*-phenyl trifluoroacetimidate glycoside **18** was employed instead. This glycosylating agent in a mixture of methylene chloride and diethyl ether at −45°C afforded 83% yield of tetrasaccharide **21** containing the α-linked glucose. Glucose building block **18** was prepared from known alcohol **16** (van Steijn et al., 1992) via the procedure that was used for the conversion of **5** to **15**. Treatment of tetrasaccharide **21** with triethylamine resulted in cleavage of the Fmoc group and liberation of the hydroxyl group, ready for the next glycosylation. Hexasaccharide **23** was obtained by the trimethylsilyl trifluoromethanesulfonate-catalyzed glycosylation of tetrasaccharide **22** with disaccharide building block **15**.

Hexasaccharide **23** was freed from all protecting groups via a three-step procedure. First, the *N*-trichloroacetyl groups were transformed into *N*-acetyl groups by treatment with tributyl stannane and azobisisobutyronitrile (AIBN) in toluene at 90°C (Figures 1 and 3) (Bélot and Jacquinet, 2000; Rawat et al., 2008). Subsequent saponification using potassium hydroxide in tetrahydrofurane and methanol was followed by hydrogenation using hydrogen gas and palladium on charcoal. Thereby, hexasaccharide hapten **2** was obtained (Figures 1 and 3).

The anomeric region of the ^{13}C and ^{1}H-NMR spectra of hexasaccharide **2** are compared to that of the natural polysaccharide (see Supplemental Experimental Procedures), confirming the structure of hexasaccharide **2** despite slight differences. During the preparation of this manuscript, an alternative synthesis of a closely related hexasaccharide was published (Danieli et. al, 2011) where a similar comparison of NMR spectra of synthetic hexasaccharide and natural polysaccharide was studied.

Preparation and Characterization of Oligosaccharide-Protein Conjugate

Polysaccharide vaccines provoke a T cell-independent immune response and do not induce an immunoglobulin class switch. Therefore, polysaccharides are conjugated to immunogenic carrier proteins that, unlike polysaccharides, induce a T cell-dependent immune response. The synthetic hapten **2** of the *C. difficile* glycopolymer PS-II was conjugated to the protein carrier CRM_{197}. The diphtheria toxoid CRM_{197} was chosen as a carrier because it is a constituent of licensed vaccines (Barocchi et al., 2007). A method based on the selective reaction of the primary amine with squaric acid diester (Tietze et al., 1991) was selected from the multitude of methods for conjugation of carbohydrates to proteins (Kuberan and Linhardt, 2000; Stallforth et. al, 2009). First, the amine group of the spacer moiety in hexasaccharide **2** was reacted with one of the vinylogous ester groups of 3,4-di-ethoxy-3-cyclobutene-1,2-dione in pH 7.2 phosphate buffer to form the corresponding monoamine **24** that was purified by reverse-phase HPLC (Figure 4A). The remaining ester group of monoamide **24** was subsequently coupled with the ε-amino groups of lysine on the diphtheria toxoid CRM_{197} in bicarbonate buffer at pH 9.0 to afford the neoglycoconjugate. Successful conjugation was confirmed by SDS-PAGE (Figure 4B), and the oligosaccharide/CRM_{197} ratio was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Figure 4C). The mass analysis of CRM_{197} yielded a m/z ion at 58.6 kDa. The mass spectrum of the neoglycoconjugate revealed mass peaks between 59.9 and 67.3 kDa corresponding to mono- to heptavalent glycoconjugates. On average four haptens **2** were loaded on the diphtheria toxoid.

Immunization and Monoclonal Antibodies

To test the immunogenicity of the PS-II hapten, two female C57BL/6 mice were immunized with the neoglycoconjugate. Mice were injected three times subcutaneously (s.c.) with the glycoconjugate at 2-week intervals. For each injection 15 μg protein, as determined by Bradford analysis, was used. Considering an average loading ratio of four haptens on each protein, the corresponding carbohydrate content was calculated as being 1.3 μg. The anti-hapten **2** antibody titers were monitored by glycan microarray analysis. Microarrays were designed for high-throughput analysis, such that 64 samples could be analyzed on one array with each well displaying hapten **2** and seven control sugars in quadruplicates (Figure 5A). The two immunized mice produced IgG antibodies that bound specifically to hapten **2** (Figure 5B), demonstrating that hapten **2** is immunoreactive. An increase in the level of anti-hapten **2** specific IgG antibodies over time was observed with mouse 2805.

To generate monoclonal antibodies, spleenocytes of the immunized mice were fused to myeloma cells by the traditional hybridoma technique (Köhler and Milstein, 1975). The individual hybridoma clones were screened to identify clones that produce anti-hapten **2** antibodies. Three hybridoma clones that secrete specific antibodies were obtained (Figure 5C). All three hybridoma clones were derived from mouse 2805. Although the monoclonal antibodies C2805.7 and C2805.21 bound exclusively to hapten **2,** antibody C2805.25 also interacted with glucose on the array.

Specific IgA Antibodies in Infected Hospital Patients

Given the immunogenicity of the hexasaccharide hapten **2** in mice, we wanted to know whether patients with CDAD produce antibodies against the native glycopolymer. To this end, stool supernatants of ten hospitalized patients with and without *C. difficile* infection, as confirmed by the VIDAS immunoassay (bioMérieux) that detects toxins A and B, were analyzed. Stool supernatant rather than serum was chosen because the contact site of the immune system with the cell surface glycopolymer is the intestinal mucosa. Accordingly, IgA rather than IgG antibodies are chiefly responsible for clearance of the pathogen

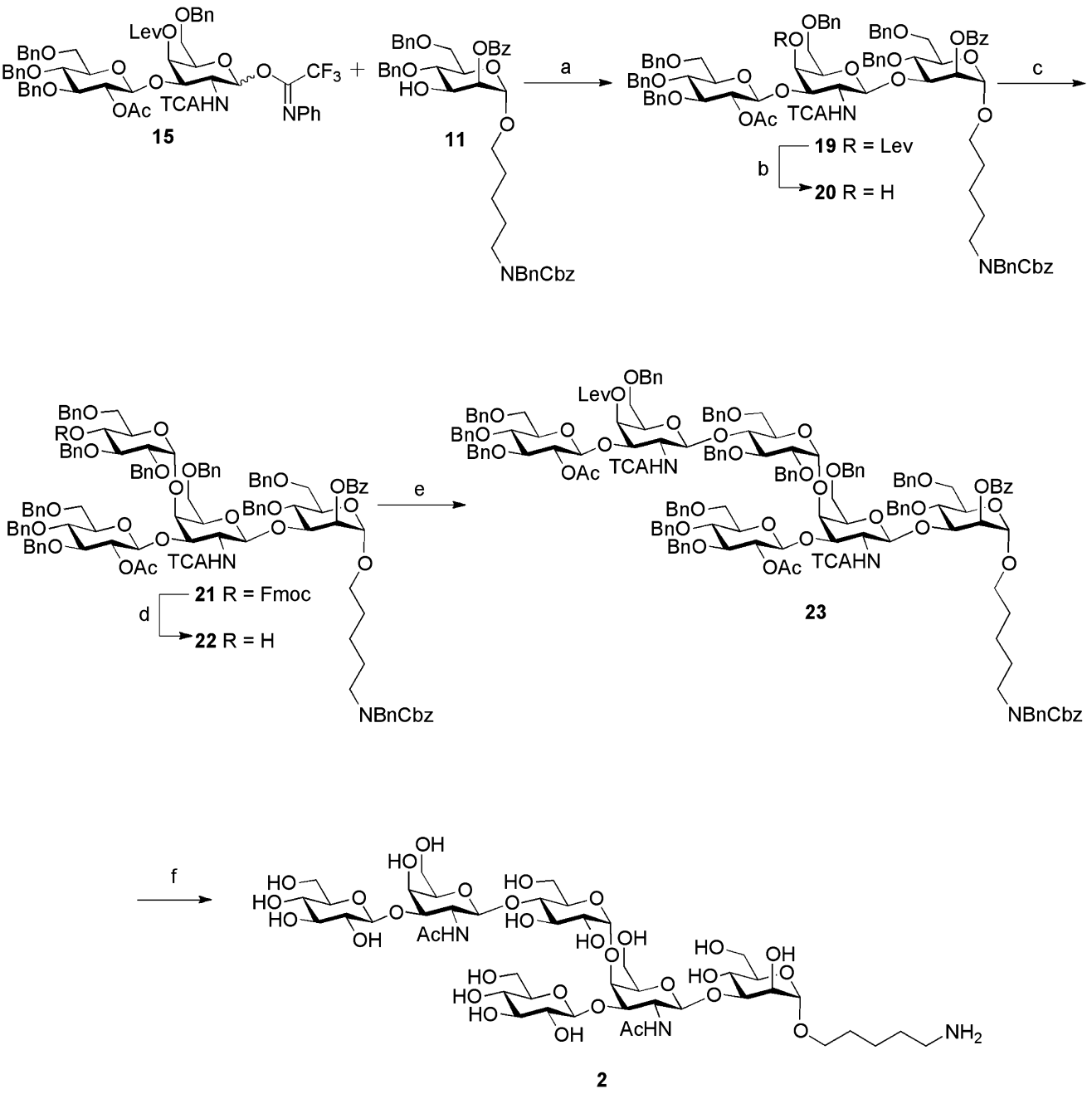

Figure 3. Synthesis of Hexasaccharide 2

Reagents and conditions: (a) TMSOTf, CH_2Cl_2, −30°C, 82%; (b) $N_2H_4 \cdot H_2O$, Py, AcOH, CH_2Cl_2, 91%; (c) **18**, TMSOTf, Et_2O, CH_2Cl_2, −45°C, 83%; (d) Et_3N, CH_2Cl_2, 85%; (e) **15**, TMSOTf, CH_2Cl_2, −30°C, 63%; (f) 1. Bu_3SnH, AIBN, toluene, 68%; 2. KOH, MeOH, THF, 86%; 3. H_2, Pd/C, AcOH, THF, MeOH, H_2O, 95%.

from the gut. In order to monitor the immune response in the epithelia against hexasaccharide **2**, the glycan arrays described above were incubated with the stool supernatants, and bound IgA antibodies were visualized. Three persons had high titers of anti-hexasaccharide **2** IgA antibodies in their stool (Figure 6). Of these three patients, two had been diagnosed with *C. difficile* toxin A/B-positive disease, whereas the third patient had a borderline VIDAS test. Low amounts of anti-hexasaccharide **2** recognizing IgA antibodies were also detected in patients 2093, 2118, and 2121, which had not been diagnosed with *C. difficile* toxin-positive disease. A possible explanation is colonization with a nontoxigenic *C. difficile* strain or previous contact with the bacterium.

DISCUSSION

We report an elegant synthesis of *C. difficile* PS-II hexasaccharide hapten that correlates with the structural assignment based on

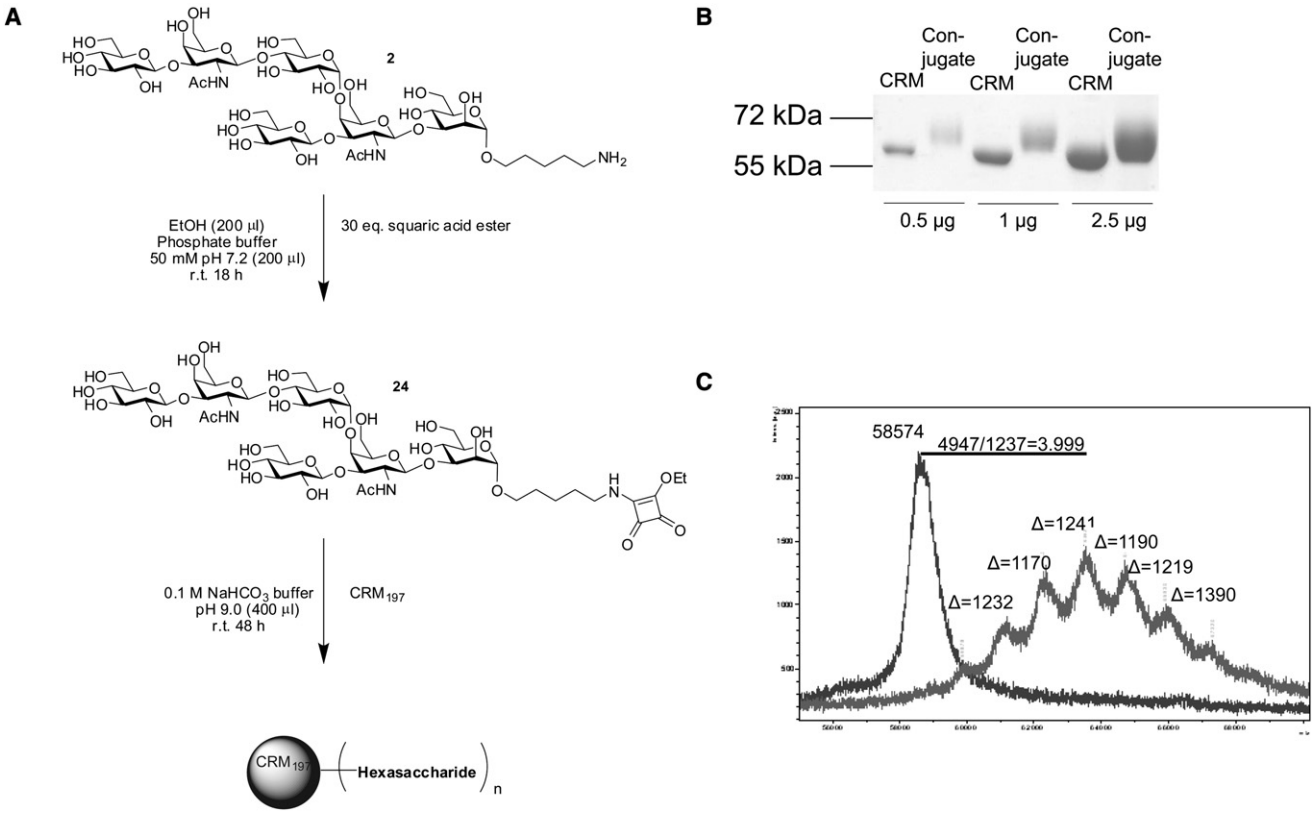

Figure 4. Conjugation and Analysis of the Hexasaccharide 2-CRM_{197} Glycoconjugate

(A) Hexasaccharide **2** was reacted with the carrier protein CRM_{197} via squaric acid route to yield a polyvalent neoglycoconjugate.

(B) SDS-PAGE analysis of the conjugation. Samples were electrophoresed on 12.5% SDS-PAGE gels and stained with Coomassie blue.

(C) MALDI-TOF MS analysis of the neoglycoconjugate. CRM_{197} with a m/z peak at 58.5 kDa; hexasaccharide **2**-CRM_{197} conjugates with m/z peaks between 59.9 and 67.3 kDa.

isolated material. The hexasaccharide was assembled from four monosaccharide building blocks using an efficient and convergent approach. A neoglycoconjugate comprising the hexasaccharide hapten and the immunogenic carrier protein CRM_{197} was obtained. The outcome of the conjugation process was monitored by MALDI-TOF MS and SDS-PAGE. Mice were immunized with the neoglycoconjugate, and IgG antibody production against hexasaccharide **2** was monitored by glycan microarray analysis. The two animals produced antibodies specific for the carbohydrate hapten, one of which showed a gradual increase of the antibody's affinity/concentration over the immunization period.

High-throughput carbohydrate microarray analysis served as a fast method to detect antibodies in murine sera, hybridoma supernatant, and human excrement. Active ester conjugation chemistry allowed for facile immobilization of the amine-terminated synthetic hexasaccharide antigen to glass slides. In addition to hexasaccharide **2**, seven control carbohydrates were printed onto the microarray slides, and the entire array was stable for more than 1 year. Carbohydrate microarray analysis gave a detailed picture of the presence of antibodies, antibody affinity and concentration, as well as cross-reactivity.

Using the microarrays, we detected specific anti-hexasaccharide hapten **2** IgA antibodies in the stool supernatants of hospital patients. Two patients with significantly increased *C. difficile* toxin A and B levels and one patient with a borderline test displayed high amounts of highly specific anti-hexasaccharide **2** IgA antibodies in their excrement. These observations suggest that native glycopolymer PS-II exposes antigenic determinants that are the targets of the immune response induced by some patients infected with *C. difficile*. Antibodies in stool are subject to different dilutions depending on the amount of daily elimination; therefore, small variations in the concentrations of the individual samples are likely. The three false-negative results may be explained by the fact that these individuals were infected with *C. difficile* strains that do not express PS-II. For the strains prevalent in European hospitals (Zaiss et al., 2010), the expression of PS-II is only confirmed for ribotype 027. Because ribotyping is not performed routinely in European hospitals, the genetic background of the pathogens responsible for the infections analyzed in this study remains elusive. The low binding signal recorded for three samples of patients without diagnosed *C. difficile* infection can be accounted for by latent or previous infections with bacteria of the clostridium type carrying PS-II.

In conclusion we show here that PS-II is an antigenic determinant upon infections of humans with *C. difficile*. Using a synthetic fragment of PS-II as hapten, we further demonstrate immunogenicity in mice and provide monoclonal antibodies specifically

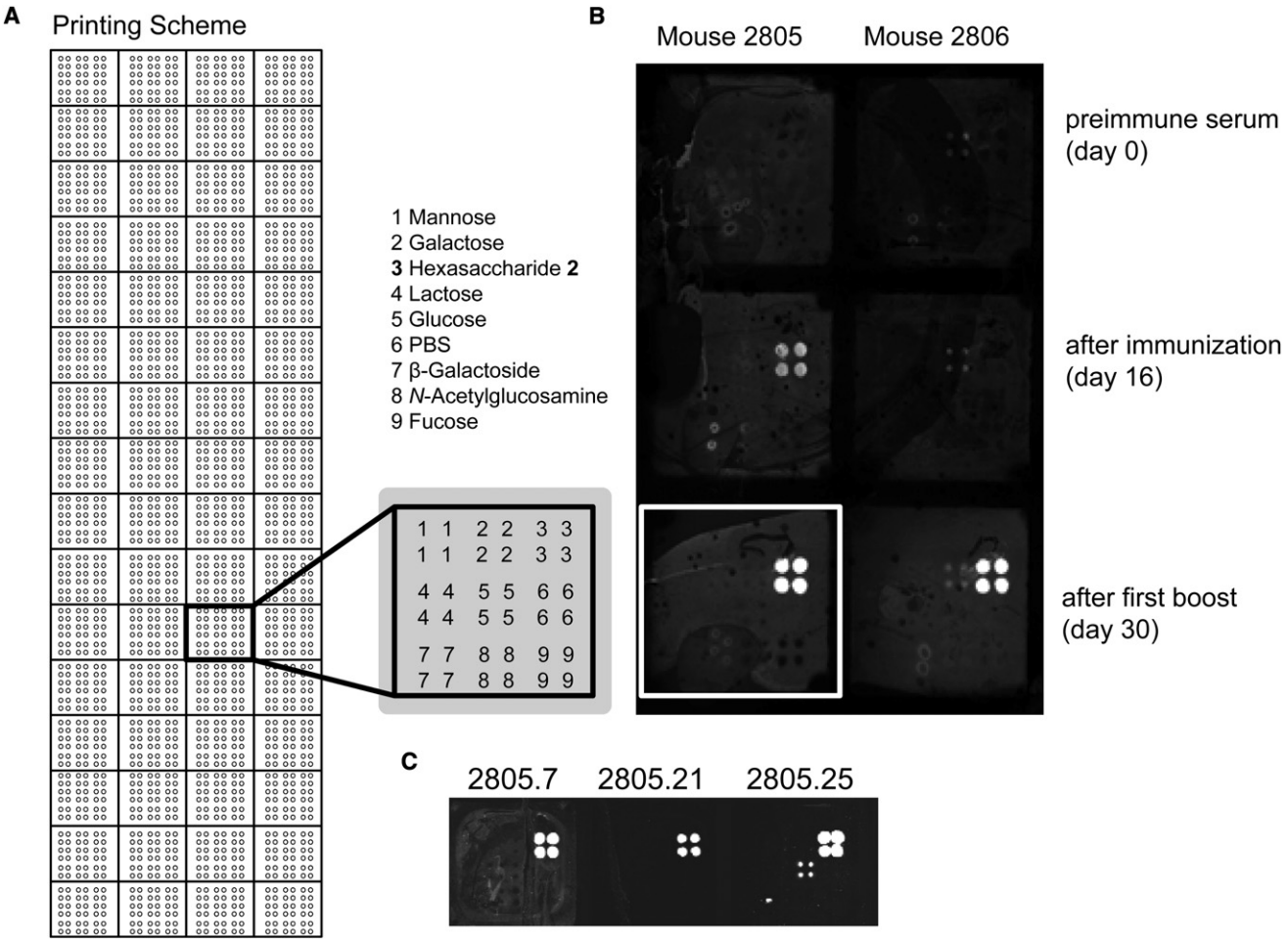

Figure 5. IgG in the Serum of Immunized Mice and Three Murine Monoclonal Antibodies Bind Hexasaccharide 2 on Glycan Microarrays

(A) Printing scheme of the glycan microarrays.

(B) IgG titers of immunized animals were analyzed before immunization (day 0), after the immunization (day 16), and after the first boost (day 30). Both mice produced IgG antibodies to hexasaccharide 2. The microarray printing pattern is shown in the gray box.

(C) Monoclonal antibodies 2805.7, 2805.21, and 2805.25 raised against the neoglycoconjugate.

interacting with the glycan hapten as determined by microarray analysis.

SIGNIFICANCE

The synthesis of a hexasaccharide fragment of a *C. difficile* cell surface polysaccharide gave access to chemically defined and structurally homogeneous material equipped with a primary amine handle. This handle allowed for conjugation of the synthetic oligosaccharide to the immunogenic carrier protein CRM_{197} and to glass surfaces to produce microarrays. The neoglycoconjugate was immunogenic in mice and produced murine monoclonal antibodies that specifically interact with the glycan hapten. The antibody-binding specificities were determined by microarray analysis. Furthermore, microarrays were used to detect IgA antibodies in the stool supernatant of infected hospital patients. The presence of anti-hexasaccharide 2 IgA antibodies in infected patients suggests a pivotal role of the PS-II in the pathogenesis of *C. difficile*-associated diseases (CDADs). Thus, both the natural polysaccharide and the synthetic substructure are further studied as potential carbohydrate conjugate vaccine candidates against *C. difficile*.

EXPERIMENTAL PROCEDURES

Chemical Synthesis

Detailed experimental procedures and characterization data for new compounds are available online; see Supplemental Experimental Procedures.

Conjugation

Diethyl squarate (7.3 μl, 51 μmol) was added to a solution of hexasaccharide C1 (2 mg, 1.7 μmol) in EtOH (0.2 ml) and phosphate buffer (0.2 ml, 50 mM [pH 7.2]) and stirred for 18 hr at room temperature. Most ethanol was removed by a stream of N_2. The mixture was purified using a HPLC Superdex size exclusion column (95:5 H_2O/EtOH) to afford a colorless solid. A solution of the squarate adduct (0.7 mg, 546 nmol) and the diphtheria toxoid CRM_{197} (Calbiochem; 0.7 mg, 11.1 nmol) in $NaHCO_3$ buffer solution (0.4 ml, 0.1 M [pH 9]) was shaken for 48 hr at room temperature. The resulting mixture was purified by ultrafiltration (30 K, Amicon; Millipore) with PBS. The protein concentration was determined by Bradford analysis (Bio-Rad).

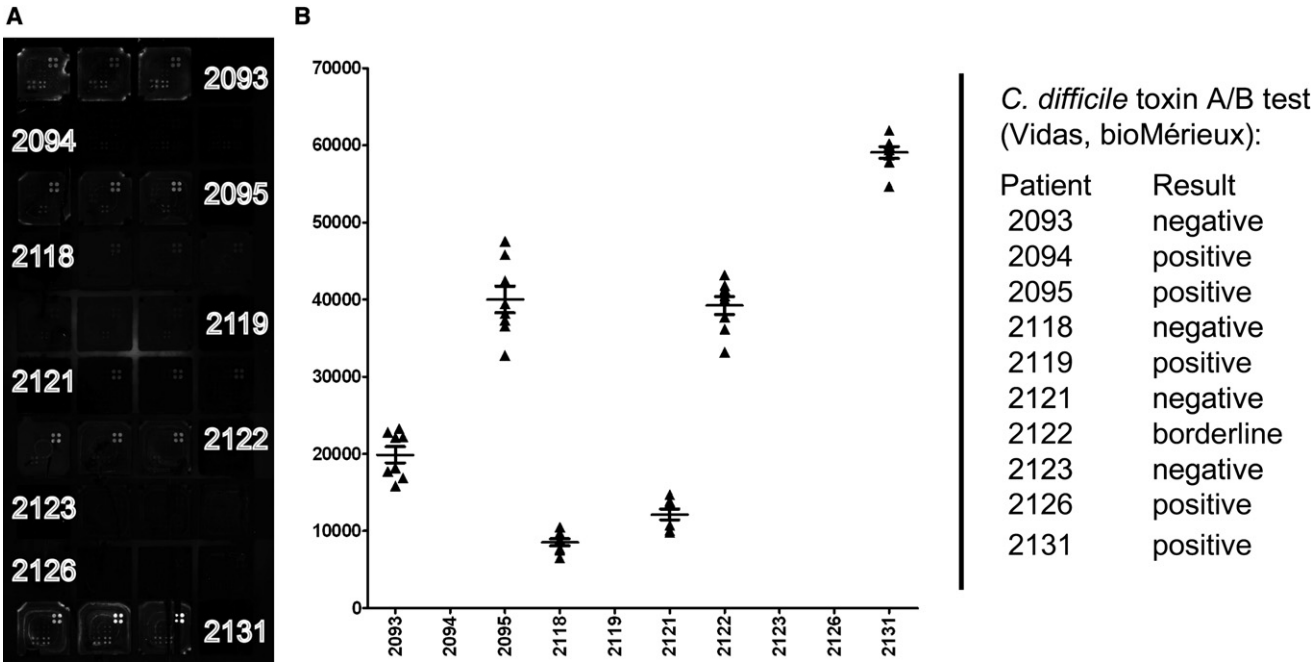

Figure 6. IgA Analysis of Stool Supernatant of Hospitalized Persons

The presence of IgA antibodies specific for hexasaccharide **2** was analyzed in stool supernatants of ten hospital patients.

(A) Glycan microarray experiment. Stool supernatant of each patient was analyzed in three adjacent microarray wells.

(B) Quantification of the fluorescence signals detected in the glycan microarray experiment. High titers of anti-hexasaccharide **2** IgA antibodies were detected in patients 2095 (positive), 2122 (borderline), and 2131 (positive). Low-intensity signals were also detected in patients 2093, 2118, and 2121 (all diagnosed negative).

SDS-PAGE

Hexasaccharide 2-CRM_{197} glycoconjugate and unconjugated CRM_{197} were dissolved in Laemmli buffer (0.125 M Tris, 20% [v/v] glycerol, 4% [w/v] SDS, 5% [v/v] β-mercaptoethanol, bromophenol [pH 6.8]) and boiled for 5 min. Samples were run in 12.5% polyacrylamide gel and stained with 0.025% (w/v) Coomassie brilliant blue R-250 in an aqueous solution containing 40% (v/v) methanol and 7% (v/v) acetic acid.

MALDI-TOF MS

Conjugation was confirmed by MALDI-TOF MS using an Ultraflex-II TOF/TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with a 200 Hz solid-state Smart beam™ laser. The mass spectrometer was operated in the positive linear mode. MS spectra were acquired over an m/z range of 4,000–80,000, and data were analyzed using FlexAnalysis® software provided with the instrument. The samples were lyophilized from 25 mM NH_4HCO_3 (pH 7.8). Sinapinic acid was used as the matrix, and samples were spotted using the dried droplet technique.

Immunizations

Two female C57BL/6 mice were immunized s.c. with 15 μg hexasaccharide **2**-CRM_{197} conjugate in complete Freund's adjuvants where 15 μg refers to the protein content. The mice were boosted twice with 15 μg hexasaccharide **2**-CRM_{197} conjugate in incomplete Freund's adjuvants in 2-week intervals. After each injection, blood was collected, and serum titers (IgG) were analyzed using microarrays. Prior to being sacrificed, mice received additional 10 μg hexasaccharide 2-CRM_{197} in PBS i.p. on 3 consecutive days.

Preparation of Clostridium Microarrays

Eight oligosaccharides bearing an amine linker were immobilized on NHS-activated slides. Besides hexasaccharide **2,** mannose, glucose, galactose, fucose, acetylglucosamine, lactose, and a β-galactoside[337] were printed in 0.5 mM concentration onto the slides. Each spot was printed in quadruplicate using a piezoelectric spotting device (S11; Scienion, Berlin, Germany). Slides were incubated in a humid chamber to complete reaction for 24 hr and stored in a desiccator until usage.

Microarray Binding Assays

A FlexWell 64 (Grace Bio-Labs, Bend, OR, USA) grid was applied to the slides. The resulting 64 wells were used for 64 individual experiments. The slide was blocked with 2.5% (w/v) BSA and 0.05% (v/v) Tween 20 in PBS for 1 hr at room temperature. Blocked slides were washed with PBS and incubated with 5% (v/v) serum in PBS or hybridoma culture supernatant for 1 hr at room temperature. Slides were washed with PBS and incubated with 10 μg/ml Alexa Fluor 594 goat anti-mouse IgG and Alexa Flour 594 goat anti-mouse IgM (both Invitrogen) secondary antibody solutions in PBS with 1% (w/v) BSA. Slides were washed with PBS and centrifuged to dryness. Slides were scanned using a GenePix 4300A scanner (Bucher Biotec, Basel, Switzerland) and evaluated using the GenePix Pro 7 software (Bucher Biotec).

Monoclonal Antibody Purification

Supernatant of the hybridoma clones was filtered through a 0.2 μm filter. The supernatant was mixed 1:1 with binding buffer (0.1 M NaP, 0.15 M NaCl [pH 7.4]) and loaded onto a Midi Protein G spin column (Proteus, Oxford, UK). The spin column was washed twice with binding buffer. Subsequently, the IgG was eluted with elution buffer (0.2 M glycan/HCl [pH 2.5]) and immediately neutralized with 1 M Tris/HCl (pH 9). The eluted antibody solution was purified by ultrafiltration (100 K, Amicon; Millipore) with PBS containing 0.01% (w/v) sodium azide. Protein-stabilizing cocktail (Pierce, Rockford, IL, USA) was added to the concentrated antibody solution, and the protein concentration was determined by Bradford analysis (Bio-Rad).

Analysis of Stool Supernatant

A FlexWell 64 (Grace Bio-Labs) grid was applied to the slides. The wells were blocked with 2.5% (w/v) BSA and 0.05% (v/v) Tween 20 in PBS for 1 hr at room temperature. Blocked slides were washed with PBS and incubated with 20 μl stool supernatant of ten hospitalized persons (Charité, Berlin) for 1 hr at room temperature. Slides were washed with PBS and incubated with 10 μg/ml goat

anti-human IgA FITC Conjugate (Invitrogen) secondary antibody solutions in PBS with 1% (w/v) BSA. Slides were washed with PBS and centrifuged to dryness. Slides were scanned using a GenePix 4300A scanner and evaluated using the GenePix Pro 7 software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2011.03.009.

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