Synthetic β -(1 $\rightarrow$ 6)-Linked N-Acetylated and Nonacetylated Oligoglucosamines Used To Produce Conjugate Vaccines for Bacterial Pathogens[†]

Marina L. Gening,¹ Tomás Maira-Litrán,^{2,‡} Andrea Kropec,^{3,‡} David Skurnik,^{2,‡} Martha Grout,² Yury E. Tsvetkov,¹ Nikolay E. Nifantiev,^{1*} and Gerald B. Pier^{2*}

N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky Prospect, 47, 119991 Moscow, Russia¹; Channing Laboratory, Brigham and Women's Hospital, Harvard Medical School, 181 Longwood Avenue, Boston, Massachusetts²; and Division of Infectious Diseases, Department of Medicine, University Hospital Freiburg, Hugstetter Str. 55, 79106 Freiburg, Germany³

Received 25 September 2009/Returned for modification 26 October 2009/Accepted 19 November 2009

Vaccines for pathogens usually target strain-specific surface antigens or toxins, and rarely is there broad antigenic specificity extending across multiple species. Protective antibodies for bacteria are usually specific for surface or capsular antigens. β -(1 $\rightarrow$ 6)-Poly-N-acetyl-D-glucosamine (PNAG) is a surface polysaccharide produced by many pathogens, including *Staphylococcus aureus*, *Escherichia coli*, *Yersinia pestis*, *Bordetella pertussis*, *Acinetobacter baumannii*, and others. Protective antibodies to PNAG are elicited when a deacetylated glycoform (deacetylated PNAG [dPNAG]; <30% acetate) is used in conjugate vaccines, whereas highly acetylated PNAG does not induce such antibodies. Chemical derivation of dPNAG from native PNAG is imprecise, so we synthesized both β -(1 $\rightarrow$ 6)-D-glucosamine (GlcNH₂) and β -(1 $\rightarrow$ 6)-D-N-acetylglucosamine (GlcNAc) oligosaccharides with linkers on the reducing termini that could be activated to produce sulfhydryl groups for conjugation to bromoacetyl groups introduced onto carrier proteins. Synthetic 5-mer GlcNH₂ (5GlcNH₂) or 9GlcNH₂ conjugated to tetanus toxoid (TT) elicited mouse antibodies that mediated opsonic killing of multiple *S. aureus* strains, while the antibodies that were produced in response to 5GlcNAc- or 9GlcNAc-TT did not mediate opsonic killing. Rabbit antibodies to 9GlcNH₂-TT bound to PNAG and dPNAG antigens, mediated killing of *S. aureus* and *E. coli*, and protected against *S. aureus* skin abscesses and lethal *E. coli* peritonitis. Chemical synthesis of a series of oligoglucosamine ligands with defined differences in N acetylation allowed us to identify a conjugate vaccine formulation that generated protective immune responses to two of the most challenging bacterial pathogens. This vaccine could potentially be used to engender protective immunity to the broad range of pathogens that produce surface PNAG.

The continued threat from antibiotic-resistant microbial pathogens such as multidrug-resistant *Staphylococcus aureus* and *Escherichia coli* and the ongoing difficulty in adequately preventing and treating infectious diseases caused by such pathogens have driven the quest for more effective preventative and therapeutic approaches to infection. Vaccination, when it works, not only dramatically decreases infection and illness (35), but also has shown itself to be capable of eliminating endemic transmission of diseases such as polio, measles, and rubella from the United States and has eliminated smallpox worldwide (35). Bacterial surface or capsular antigens, which are commonly synthesized as polysaccharides and less commonly as proteins, represent the best-established targets

for engendering protective immunity by vaccination. Conjugating surface polysaccharides to carrier proteins greatly enhances the immunogenicity and effectiveness of the polysaccharides (40). Highly successful conjugate vaccines targeting the capsular polysaccharides (CPs) of *Streptococcus pneumoniae* (5), *Haemophilus influenzae* type b (38), and *Neisseria meningitidis* (40) have been produced and licensed for human use, with a major impact in reduction of disease due to these bacterial pathogens. Significant advances, including those from human trials, have been made for polysaccharide conjugate vaccines for *Salmonella enterica* serovar Typhi (20), group B streptococcus (4), and *E. coli* O157 (1).

A promising target for vaccine development is a surface polysaccharide produced by a broad range of common pathogens and designated poly-N-acetylglucosamine (PNAG), a β -(1 $\rightarrow$ 6)-linked polymer of N-acetyl-D-glucosamine (GlcNAc) (22) with some proportion of the amino groups lacking acetate substituents. The basic chemical properties of PNAG were described by Mack et al. (21), who referred to the material as the polysaccharide intercellular adhesin. Among important bacterial pathogens, PNAG is known to be produced by *S. aureus* and *Staphylococcus epidermidis* (23, 25, 26), *E. coli* (13, 42), *Bordetella pertussis* and *Bordetella parapertussis* (29, 36), *Aggregatibacter actinomycetemcomitans* (15), *Acinetobacter* spp. (8), and *Yersinia pestis* (10, 12). Based on genetic homology,

* Corresponding author. Mailing address for Nikolay E. Nifantiev (for chemistry): N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky Prospect, 47, Moscow B-334, 119991 Russia. Phone and fax: 007-499-1358784. E-mail: nen@ioc.ac.ru. Mailing address for Gerald B. Pier (for biology): Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, 181 Longwood Ave., Boston, MA 02115. Phone: (617) 525-2269. Fax: (617) 525-2510. E-mail: gpier@channing.harvard.edu.

[†] Supplemental material for this article may be found at <http://iai.asm.org/>.

[‡] These authors contributed equally to this work.

[§] Published ahead of print on 30 November 2009.

FIG. 1. Introduction of thiol linkers into the carbohydrate ligands. Boldfaced numbers indicate structures referred to in descriptions of oligosaccharide synthesis in Materials and Methods and in Fig. 2. Ac, acetyl; BZ, benzoyl; Phth, phthaloyl; Z, benzylcarbonyl; Su, succinimidyl; Pfp, pentafluorophenyl; Et₃N, triethylamine; DTT, dithiothreitol; Ac₂O, acetic anhydride.

loci likely encoding PNAG biosynthetic proteins are found in *Burkholderia cenocepacia* and *Klebsiella pneumoniae*. Prior work has shown that antibodies to PNAG conjugated to a protein carrier can mediate *in vitro* opsonic killing and protect mice from *S. aureus* (23, 26) and *E. coli* (7) infections, but such immunity can be engendered only by first removing the majority of the acetates from the PNAG polymer to produce deacetylated PNAG (dPNAG). These findings indicate that the immunodominant epitopes on native PNAG elicit nonopsonic, nonprotective antibodies and that antibodies to the core or backbone epitopes have superior opsonic and protective properties, due likely to enhanced deposition of opsonically active fragments of the third component of complement, C3b (16).

While conjugate vaccines comprising highly but not completely deacetylated forms of PNAG appear to be effective at providing protective immunity in animal studies, the lack of definition of the chemical composition of dPNAG and the need to produce it by chemical deacetylation of highly acetylated PNAG, resulting in variability in the final composition, limit the conclusions that can be drawn about optimal vaccine formulation. Native PNAG (>90% acetylated) has a certain amount of deacetylated β -(1 $\rightarrow$ 6)-D-glucosamine (GlcNH₂) units but whether they are grouped together or interspersed throughout the molecule is not known, nor is it known if

preparations of either native PNAG or dPNAG contain a proportion of molecules with low levels of acetylation among a greater population of highly acetylated molecular species. To develop optimal vaccines that generate protective antibodies, the relative numbers of GlcNH₂ units and their spacing will need to be determined, and this will not be possible by chemical deacetylation, which would randomly change GlcNAc units to GlcNH₂ units.

To define more precisely the immune responses elicited by different epitopes on the PNAG molecule, oligoglucosamines containing either 5- or 9-mer fully acetylated monosaccharides (5GlcNAc or 9GlcNAc) or 5- or 9-mer fully nonacetylated monosaccharides (5GlcNH₂ and 9GlcNH₂) were conjugated to a protein carrier (tetanus toxoid [TT]) and used to immunize mice and rabbits. The fully acetylated oligosaccharides elicited high titers of nonopsonic antibodies in mice, whereas the fully nonacetylated oligosaccharides elicited highly active opsonic antibodies in mice and rabbits, with the antibodies from the latter species showing excellent passive protective efficacy against *S. aureus* skin infections and *E. coli* peritonitis.

MATERIALS AND METHODS

Bacterial strains used. The *S. aureus* strains used were CP8 strain MN8 (18), CP5 strain Newman (3), nontypeable (NT) USA 300 methicillin-resistant *S. aureus* (MRSA) strains LAC (27) and SF8300 (9), and an isogenic set of three

FIG. 2. General scheme of conjugation and compositions of glycoconjugates used for immunization. Boldfaced numbers 7, 8, 9, and 10 refer to structures with the same numbers depicted in Fig. 1. Ac, acetyl; aq., aqueous.

strains derived from CP5 strain Reynolds wherein two genetically engineered derivatives were produced to express either no CP antigen or the CP8 antigen in place of the native CP5 antigen (43). The latter three strains were kindly provided by Jean Lee, Boston, MA. Mutants of *S. aureus* strains MN8 and Newman lacking the *ica* locus for the biosynthesis of PNAG (*ica::ter*) have been described previously (19). Clinical urinary tract infection (UTI) isolates of *E. coli* producing PNAG (strains J and P) and a strain unable to synthesize PNAG (strain H) have been described previously (7).

Synthesis of oligosaccharides. Production of the thiol-derivatized 5GlcNH₂ or 9GlcNH₂ and 5GlcNAc or 9GlcNAc oligosaccharides is graphically depicted in Fig. 1. The supplemental material also contains additional experimental details and nuclear magnetic resonance spectral data to confirm the structures produced at each synthetic step. Amino groups in the spacers of the oligosaccharides (11) were selectively deprotected and acylated using linker reagents 1 and 2 (Fig. 1) to give corresponding pentasaccharide 5 and nonasaccharide 6. Introduction of the linkers was confirmed by nuclear magnetic resonance and mass spectral data (see the supplemental material). After total deprotection using hydrazine hydrate in boiling ethanol, oligoglucosamine ligands with free amino groups (ligands 7 and 9) were obtained. According to the spectral data, both ligands 7 and

9 existed as mixtures of the sulfhydryl compounds and corresponding disulfides. The latter arose upon spontaneous oxidation of SH derivatives with atmospheric oxygen. Although the rates of oxidation of these two compounds were different, we considered ligands 7 and 9 to be disulfides and used suitable reducing agents for reliable exposure of the sulfhydryl group for further derivatization. Thus, with the use of dithiothreitol and acetic anhydride, N- and S-acetylated derivatives 8 and 10 (Fig. 1) were produced.

Preparation of the conjugate vaccines. The production of the conjugate vaccines is graphically depicted in Fig. 2. Oligosaccharides 7 and 9 (Fig. 1) (1.5 mg in a 100- μ l mixture of 0.1 M sodium phosphate, 0.15 M NaCl, and 10 mM EDTA, pH 8.0) were treated with washed Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) disulfide reducing gel (200 μ l of a 50% slurry in water). After incubation on a rotor rack at 22 to 24°C for 45 min, SH-derivatized oligosaccharides were separated from the gel by centrifugation, the immobilized TCEP was washed three times with 100 μ l of the same pH 8.0 buffer, and supernatants were combined.

TABLE 1. Conjugates and doses used to immunize mice		
Mouse group	Conjugate or preparation	Dose (μ g/mouse)/week ^a
1	5GlcNH ₂ -TT	0.5
2	5GlcNH ₂ -TT	2.5
3	5GlcNH ₂ -TT	10
4	9GlcNAc-TT	0.5
5	9GlcNAc-TT	2.5
6	9GlcNAc-TT	10
7	5GlcNH ₂ + TT ^b	10
8	5GlcNAc + TT ^b	10
9	9GlcNH ₂ -TT	0.5
10	9GlcNH ₂ -TT	2.5
11	9GlcNH ₂ -TT	10
12	9GlcNAc-TT	0.5
13	9GlcNAc-TT	2.5
14	9GlcNAc-TT	10

^a Doses are based on micrograms of hexosamine. Mice received three doses total.

^b Mix of oligosaccharide and TT.

FIG. 3. Immune responses of mice immunized with GlcNH₂-TT or GlcNAc-TT conjugate vaccines. (A) ELISA-determined titers of antibodies to PNAG. (B) ELISA-determined titers of antibodies to dPNAG. Sera were obtained from mice immunized with the indicated amounts of carbohydrate equivalents of conjugates 5GlcNH₂-TT, 9GlcNH₂-TT, 5GlcNAc-TT, or 9GlcNAc-TT. Bars represent means.

Aliquots of 2.1 ng of the fully acetylated 5GlcNAc and 9GlcNAc oligomers terminating with an *S*-acetyl group (structures 8 and 10 in Fig. 1) were each dissolved in 200 μ l of a 7% aqueous ammonia solution, and the solutions were kept at 22 to 24°C for 1 h and then lyophilized. Lyophilized oligosaccharide was immediately dissolved in a 400- μ l mixture of 0.1 M sodium phosphate, 0.15 M NaCl, and 10 mM EDTA, pH 8.0.

To activate the carrier protein, TT (4 mg) was diluted with a 400- μ l mixture of 0.1 M sodium phosphate, 0.15 M NaCl, and 10 mM EDTA, pH 7.2, after which a solution of *N*-hydroxysuccinimidyl-3-(bromoacetamido)propionate (SBAP; 2.6 mg in 80 μ l of dimethyl sulfoxide) was added and the dilution was incubated for 2 h at 22 to 24°C. Unreacted SBAP was removed using a PD-10 column with a mixture of 0.1 M sodium phosphate, 0.15 M NaCl, and 10 mM EDTA, pH 8.0, and the eluate was concentrated to 400 μ l. The SH-activated oligosaccharides were immediately combined with modified TT protein (400 μ l in pH 8.0 buffer), the mixture was stirred for 18 to 24 h at 22 to 24°C, and then the conjugate was separated from uncoupled components by gel filtration with a Superose 6 prep-

grade column. Fractions containing oligosaccharide-TT conjugates were pooled, concentrated, and stored frozen at -20°C .

Chemical analysis of the conjugate vaccines. Conjugate vaccines were analyzed for oligosaccharide contents by using a hexosamine assay (37), with the corresponding free oligosaccharide used as a standard. The protein contents were measured with the Bradford assay (6) using TT as a standard.

Antibody production using oligosaccharide conjugates. The studies were approved by the Harvard Medical Area Institutional Animal Care and Use Committee. Mice were immunized subcutaneously (s.c.) with a total of three doses of 0.5, 2.5, or 10 μ g (carbohydrate content) of the 5- or 9-mer GlcNAc-TT or GlcNH₂-TT vaccines given once a week for 3 weeks. Two groups received an unconjugated mixture of either 5GlcNAc and TT or 5GlcNH₂ and TT s.c. Sera were obtained prior to injection and then weekly for 3 weeks after the last immunization. Rabbits were immunized s.c. with 10 μ g (carbohydrate content) of the 9GlcNH₂-TT conjugate twice, 1 week apart, along with an equivalent volume of Sepeal adjuvant. In the third week, rabbits were immunized three times on

FIG. 5. Immunologic activities of rabbit antisera to 9GlcNH₂-TT conjugate vaccine. (A) ELISA-determined titers of antibodies to PNAG, dPNAG, 9GlcNH₂, and 9GlcNAc obtained 2 and 6 weeks after the last immunization. (B) Opsonic killing of different *S. aureus* strains by rabbit antisera to 9GlcNH₂-TT obtained 6 weeks after the last immunization. (C) Opsonic killing of two PNAG-positive *E. coli* strains (J and P) but not a PNAG-negative strain (*E. coli* H) by rabbit antisera to 9GlcNH₂-TT obtained 6 weeks after the last immunization. The level of activity of preimmune sera was subtracted out. C', complement. Bars represent means.

FIG. 6. SPR analysis of the binding of the indicated antigen at the indicated nanomolar concentration to rabbit IgG isolated by protein G affinity chromatography from an antiserum raised against the 9GlcNH₂-TT conjugate vaccine.

alternate days with 10 μ g (carbohydrate content) of the conjugate given intravenously in saline. Blood was taken 2 weeks after the final immunization and again 1 month later.

Antibody analysis. Analysis of the binding of antibody by an enzyme-linked immunosorbent assay (ELISA) to native PNAG or dPNAG antigens was conducted as described previously (23), as was an opsonic killing assay (16, 23). Affinity associations between the rabbit antibody and 11GlcNH₂ and 11GlcNAc oligosaccharides (11), as well as native PNAG and dPNAG prepared from native PNAG (~20% acetylation), were evaluated by using surface plasmon resonance (SPR) with a BIAcore 3000 system (BIAcore AB, Uppsala, Sweden). IgG was purified from the immune sera raised against the 9GlcNH₂-TT conjugate vaccine by using protein G immobilized on agarose beads, and then the IgG was bound to a CM5 sensor chip by utilizing an amine-coupling kit according to the instructions of the manufacturer (BIAcore AB). Measurements were made using native PNAG and dPNAG with an average molecular mass of 100 kDa and defined concentrations of 11GlcNAc or 11GlcNH₂ oligosaccharides.

Animal protection studies. Skin infection studies were approved by the Regierungspräsidentium Freiburg for the University Hospital, Freiburg, Germany. Abscess formation in mice was induced by s.c. infection with *S. aureus* and Cytodex 1 microcarrier beads (131 to 220 μ m; Sigma) as described previously (14). *S. aureus* strains were grown in 1 liter of Trypticase soy broth (TSB) for 18 to 24 h at 37°C with shaking at 200 rpm, cultures were centrifuged, and bacterial cells were suspended in 10 ml of 0.9% NaCl. Aliquots were rapidly frozen and stored at -80°C. For challenge, a vial was defrosted and then the contents were diluted in TSB and mixed with an equal volume of a sterile slurry of the dextran beads. Twenty-four hours prior to infection, mice were injected intraperitoneally (i.p.) with 200 μ l of normal rabbit serum (NRS) or immune rabbit serum. Doses of 100 μ l of the challenge preparation were injected into both flanks of each mouse, and after 72 h, the animals were euthanized and the abscess materials were removed, dissected, homogenized, and diluted in TSB for bacterial enumeration.

The peritonitis infection model using *E. coli* was approved by the Harvard Medical Area Institutional Animal Care and Use Committee and followed the previously described protocol (7). *E. coli* was grown for 18 to 24 h in TSB plus 1% glucose, washed, and resuspended at a concentration of 5×10^8 CFU/ml in phosphate-buffered saline. An inoculum of 0.2 ml (10^8 CFU of *E. coli*) was injected i.p. Twenty-four hours before infection, mice were given 0.4 ml of either NRS or serum with antibody raised against 9GlcNH₂-TT. Moribund mice were sacrificed and counted as dead for the purposes of these experiments. Spleens from euthanized mice were recovered and cultured on MacConkey agar to ascertain that *E. coli* bacteria had disseminated from the initial site of infection.

Statistical analysis. Statistical analyses were performed using the Prism software. The Mann-Whitney nonparametric U test for two-sample comparisons was used to analyze paired data. Nonparametric analysis of variance was used for multigroup comparisons, and the Dunn procedure was used for *post hoc* pairwise comparisons.

RESULTS

Characterization of the oligoglucosamine conjugate vaccines. The compositions of the conjugate vaccines are shown in Fig. 2. Conjugated oligosaccharides were readily separated from unconjugated oligosaccharides by column chromatography. However, it was not possible to determine if there were unconjugated molecules of TT in the final product, as the size differences between conjugated and unconjugated forms of TT were too small for reliable separation of these forms by chromatography.

Immune responses of mice to oligoglucosamine-protein conjugate vaccines. C3H/HeN mice were immunized with the con-

FIG. 7. Protective efficacy of rabbit antibody to 9GlcNH₂-TT conjugate vaccine against skin abscess formation by *S. aureus*. Antisera (200 μ l) were administered i.p. 24 h prior to injection of skin with the indicated *S. aureus* strain mixed with 133- to 220- μ m dextran beads. Points indicate numbers of CFU per individual abscess, and lines indicate the group medians. *P* values were calculated by the Mann-Whitney U test. (A) Results following challenge with three doses (indicated on the x axis) of *S. aureus* LAC (NT USA 300 strain). (B) Results following challenge with wild-type *S. aureus* Newman (CP5) in comparison with the lack of efficacy of antibody against the *S. aureus* Newman *ica::tet* mutant unable to synthesize PNAG. (C) Results of the same type of comparison described in panel B for *S. aureus* MN8 (CP8). (D) In mice given NRS, wild-type (WT) *S. aureus* MN8 and Newman produce more CFU per abscess than do the corresponding *ica::tet* mutants.

jugate vaccines by using the schedule and doses shown in Table 1. Mice immunized with 10- μ g doses, based on the carbohydrate content, of either the 5GlcNH₂-TT or 9GlcNH₂-TT conjugate made robust responses with IgG antibodies that bound to both native PNAG and dPNAG (Fig. 3). Lower doses of the GlcNH₂-TT vaccines produced lesser immune responses. Responses peaked 3 weeks after the final dose. Mice immunized with the 5- or 9-mer GlcNAc-TT conjugates made excellent responses to the native PNAG molecule (Fig. 3A), and responses were already optimal 1 week after the last immunizing dose (data not shown). However, no binding of antibody to dPNAG was detected in the sera of mice immunized with either of the GlcNAc-TT conjugates (Fig. 3B). No immune response to either PNAG or dPNAG was detected in the sera of mice immunized with a mix of TT and either the 5GlcNH₂ or the 5GlcNAc oligosaccharide (data not shown).

When mouse sera were tested for opsonic killing activity in the presence of human polymorphonuclear leukocytes and rabbit complement, the animals immunized with either of the GlcNH₂-TT conjugates clearly had opsonically active antibod-

ies that mediated killing of *S. aureus* CP8 strain MN8 (Fig. 4A). The antibody raised against either of the GlcNAc-TT conjugates had no killing activity (Fig. 4A). When tested against two other *S. aureus* strains, Newman (CP5) and a USA 300 strain, LAC (NT), the antibodies raised against the two GlcNH₂-TT conjugates were again opsonically active (Fig. 4B). When either 11GlcNH₂ or 11GlcNAc oligosaccharides were included in the opsonic assay with sera raised against either 5GlcNH₂-TT or 9GlcNH₂-TT conjugates, the nonacetylated oligomer strongly inhibited opsonic killing whereas the fully acetylated oligomer did not (Fig. 4C). Overall, the synthetic oligomers showed that nonacetylated glycoforms elicited the best opsonic killing activity with specificity for epitopes on the homologous immunizing oligosaccharide antigen.

Rabbit immune response to 9GlcNH₂-TT conjugate vaccine. As the nonacetylated glycoform induced the desired opsonic killing activity in mice, we used the 9GlcNH₂-TT conjugate to immunize rabbits and test binding, opsonic killing, and protective activities of antibodies. Sera obtained 2 and 6 weeks after the last injection had high titers of antibodies to native PNAG

and dPNAG isolated from *S. aureus*, as well as to the immunizing 9GlcNH₂ oligosaccharide, but failed to bind to the fully acetylated 9GlcNAc oligosaccharide (Fig. 5A). Thus, these antibodies bound to epitopes not requiring the presence of acetates on PNAG but notably also bound to the highly acetylated native form of the polysaccharide that is the dominant glycoform on bacterial surfaces. Antibodies in postimmunization rabbit antisera mediated dose-dependent opsonic killing of a variety of *S. aureus* strains with different CP types (Fig. 5B), as well as two USA 300 MRSA strains lacking CP antigens (strains LAC and SF8300). Similarly, these antibodies mediated opsonic killing of two *E. coli* strains previously shown to produce PNAG but not a third strain *E. coli* lacking the *pga* genes encoding the biosynthetic enzymes for PNAG (Fig. 5C) (7).

SPR analysis to determine the K_a and K_d (association and dissociation constants) of the IgG antibodies raised against 9GlcNH₂-TT showed no binding of the antibodies to the fully acetylated 11GlcNAc molecule (Fig. 6B). Binding to the 11GlcNH₂ oligosaccharide (Fig. 6A) indicated a K_a of 3.2×10^8 and a K_d of 3.1×10^{-9} . Estimates of the K_a and K_d for binding to PNAG were 1.9×10^7 and 5.2×10^{-8} , respectively, and those for binding to dPNAG (~20% acetylated) were 4.2×10^{10} and 2.4×10^{-11} , respectively (Fig. 6C and D). However, because of the heterogeneous nature of the polysaccharide molecules and indications of multiple binding sites on the dPNAG molecule from the SPR analysis, the K_a and K_d values for the polysaccharides are, at best, approximations. There was no binding of antibody raised against 9GlcNH₂-TT to the alginate polysaccharide antigen from *Pseudomonas aeruginosa* (39) (Fig. 6E).

Protective efficacy against *S. aureus* in a murine skin abscess model. In a murine model of skin abscesses elicited by inoculating mice s.c. with bacteria, along with small (133- to 220- μ m) dextran beads (14), injection with 200 μ l of immune sera 24 h prior to infection resulted in highly significant reductions in the numbers of bacterial CFU per abscess produced by three different infectious doses of MRSA strain LAC compared with those in animals given NRS (Fig. 7A). Similarly, the antisera also significantly reduced the numbers of CFU per abscess formed by CP5 strain Newman (Fig. 7B) and CP8 strain MN8 (Fig. 7C). When the sera were tested against mutants of strains Newman and MN8 unable to make PNAG due to the deletion of the *ica* biosynthetic locus (*ica::tet*), there was no significant ($P > 0.05$) protective efficacy (Fig. 7B and C). Of note, comparisons of the numbers of CFU per abscess in mice treated 24 h prior to infection with NRS and then infected with either wild-type *S. aureus* or Δ ica *S. aureus* strain MN8 or Newman showed that the mutant strains produced significantly lower numbers of CFU per abscess than the wild-type strains 72 h postinfection (Fig. 7D). This outcome suggests that the loss of PNAG resulted in a reduction of the ability of *S. aureus* to grow or survive within the infected skin. Finally, as a further test of the antisera's specificities, we adsorbed either nonimmune or 9GlcNH₂-TT immune sera with whole cells of PNAG-producing *S. aureus* strain MN8 or strain MN8 *ica::tet* (19) and tested protective efficacy in the skin abscess model. Sera adsorbed with strain MN8 *ica::tet* bacterial cells retained antibody binding to PNAG (data not shown) and also retained significant ability to reduce the numbers of CFU per abscess compared

FIG. 8. Adsorption of antisera to 9GlcNH₂-TT conjugate vaccine with PNAG-producing *S. aureus* removes protective efficacy in the skin infection model. Rabbit antisera raised against the 9GlcNH₂-TT conjugate were adsorbed (Ads) with either *ica::tet* PNAG-negative *S. aureus* strain MN8 or PNAG-producing wild-type strain MN8 and then injected into mice that were subsequently infected with *S. aureus* MN8. The numbers of CFU per abscess at 72 h postinfection were then determined. Points indicate individual counts from abscess samples, and horizontal lines indicate group medians. P values were determined by Kruskal-Wallis nonparametric analysis of variance (ANOVA), and pairwise comparisons were performed by the Dunn procedure. $P < 0.05$ for comparisons of results for the group receiving antisera to 9GlcNH₂-TT adsorbed with *S. aureus* strain MN8 *ica::tet* (Δ ica) and results for the other three groups.

with those in mice receiving similarly adsorbed NRS (Fig. 8). In contrast, adsorption of the antisera to 9GlcNH₂-TT with PNAG-producing wild-type strain MN8 reduced levels of antibody to PNAG (data not shown) and removed the protective efficacy, as the number of CFU per abscess was no different than that in mice given NRS adsorbed with *S. aureus* strain MN8 (Fig. 8).

Protective efficacy against *E. coli* peritonitis. Prior results have shown that most, but not all, *E. coli* isolates from patients with UTIs make PNAG (7). We tested the protective efficacy of antibody to 9GlcNH₂-TT in a lethal peritonitis model of *E. coli* infection. This antibody protected all immunized mice against infection caused by two PNAG-positive *E. coli* isolates (UTI strains J and P) (Table 2), whereas all controls receiving NRS did not survive. No protection against PNAG-negative *E. coli* strain H was afforded by antibody to 9GlcNH₂-TT. These results suggest a potential for vaccination against PNAG for *E. coli* infections, although further studies are warranted.

DISCUSSION

Because of the range of bacterial pathogens that produce PNAG, this antigen is an attractive vaccine candidate for multiple, important human pathogens (7, 8, 10, 12, 24, 29, 36). However, analysis of the immune response made to PNAG among normal humans indicates that the preponderance of natural antibodies bind to highly acetylated PNAG but fail to mediate opsonic killing of or protection from *S. aureus* (17). Prior animal studies comparing the immunogenicity of highly acetylated PNAG and that of poorly acetylated (~15%) dPNAG conjugated to a nontoxic diphtheria toxoid carrier

TABLE 2. Protective efficacy of antibody to 9GlcNH₂-TT against lethal peritonitis caused by *E. coli*

<i>E. coli</i> strain used for challenge	No. of survivors/total no. of mice receiving:		<i>P</i> value (Fisher's exact test)
	Anti-9GlcNH ₂ -TT	NRS	
J (PNAG ⁺)	8/8	0/8	0.0002
P (PNAG ⁺)	8/8	0/8	0.0002
H (PNAG ⁻)	0/8	0/8	1.0

protein confirmed that the removal of acetates facilitates the induction of antibodies that bind to both native PNAG and dPNAG and are opsonic and protective against experimental *S. aureus* (23) and *E. coli* (7) infections. However, it was not clear from the prior vaccine studies if some level of acetylation on the glucosamine monomers was needed for the maximal protective immunity or if some specific pattern of acetylation had to be maintained in order to produce a protective immune response. Results obtained with the fully nonacetylated, synthetic GlcNH₂-TT conjugate vaccines showed that no acetylation is needed for generating high levels of opsonic and protective antibodies in animals, that conjugating a molecule as small as five GlcNH₂ monomers in size is sufficient for a robust immune response, and that these antibodies readily bind to highly N-acetylated PNAG, poorly acetylated dPNAG, and the nonacetylated oligosaccharides. These are critical findings, as the actual compositions of the PNAG molecules on bacterial surfaces are not precisely known and likely also vary by strain, species, and growth conditions. Thus, to target PNAG with protective antibodies, the antibodies need to bind to the molecule regardless of the level of acetylation.

It has been proposed previously that more effective vaccines against microbial pathogens could be constructed by combining multiple antigens into a single vaccine. The results obtained for GlcNH₂ oligomers synthesized with a reducing-end linker containing a reactive sulfhydryl group suggest that vaccines targeting microbes that make PNAG could be made more effective by conjugating the GlcNH₂ oligosaccharides to microbial proteins that also induce protective immunity. For example, the LcrV protein of *Y. pestis* is an outstanding target for protection against plague (28, 34), but serologic variants of this protein are known to exist among strains of *Y. pestis* circulating in central Asia (2), making it possible that such strains could evade immunity engendered by a single LcrV vaccine component. As PNAG is expressed by *Y. pestis* (12), conjugating GlcNH₂ oligomers to LcrV may enhance the protective coverage of a plague vaccine. While no formal cost analysis has been undertaken to compare the expenses of using synthetic oligomers to PNAG as opposed to PNAG isolated from bacteria as a vaccine antigen, it is our impression that the synthetic version can be produced fairly inexpensively and that, importantly, the resulting vaccine preparations will not have any microbial contaminants.

Synthetic carbohydrate vaccines are rarely studied due to the complexity of production, but conjugate vaccines containing synthetic poly(ribosyl-ribitol phosphate) provide protective immunity against *H. influenzae* type b infections and are licensed for human use (41). Pozsgay and colleagues have synthesized the *S. dysenteriae* type 1 O antigen tetrasaccharide repeat unit

and made a glycoconjugate vaccine that elicits high levels of antibodies in mice (33). Phalipon et al. (30) used monoclonal antibodies to the *Shigella flexneri* 2a O antigen to define a pentadecasaccharide representing three biological repeating units in five monomer units as a potential component of a glycoconjugate vaccine, which was recently validated as an effective vaccine for generating antibody to the 2a O antigen (31). Other synthetic oligosaccharide vaccines for a variety of pathogens are currently in various stages of development (32), indicating that more success with this technology is likely to come.

Overall, our findings indicate that small oligomers of GlcNH₂ conjugated to a carrier protein can induce high titers of opsonic antibodies that are also protective against experimental *S. aureus* skin infection and lethal peritonitis due to *E. coli*. If antibody to the nonacetylated GlcNH₂ glycoform is truly protective against the range of pathogens producing PNAG as a surface molecule, then there appears to be a high potential to use this material as a component of vaccines for humans as long as it is immunogenic and safe. Currently, further preclinical evaluations of this vaccine's protective efficacy against various PNAG-producing pathogens are ongoing to validate the utility of eventual human trials of such a preparation.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health, National Institute of Allergy and Infectious Diseases grant numbers AI46706 and AI057159 and a component of award number U54 AI057159, and grant RUB1-2639-MO-05 from the U.S. Civilian Research and Development Foundation.

The content of this report is the responsibility solely of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases or the National Institutes of Health.

G.B.P. and T.M.-L. have developed intellectual property that has been licensed for the development of PNAG- and dPNAG-based vaccines and have received consulting income, licensing fees, and royalty income from this arrangement.

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