Synthetic β-(1→6)-linked N-acetylated and non-acetylated oligoglucosamines to produce conjugate vaccines for bacterial pathogens

Running title: Synthetic PNAG-oligosaccharide conjugate vaccines

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ABSTRACT

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→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 (dPNAG, <30% acetates) 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→6)-d-glucosamine (GlcNH₂) and β-(1→6)-d-N-acetylglucosamine
(GlcNAc) oligosaccharides with linkers on the reducing terminus that could be activated to
produce sulphydryl groups for conjugation to bromacetyl groups introduced onto carrier proteins.
Synthetic 5- or 9-mer GlcNH₂ conjugated to tetanus toxoid (TT) elicited mouse antibodies that
mediated opsonic killing of multiple *S. aureus* strains, while the antibodies that were produced to
the 5- or 9-mer GlcNAc-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.
INTRODUCTION

The continued threat from antibiotic-resistant microbial pathogens such as multi-resistant *Staphylococcus aureus* and *Escherichia coli*, and ongoing difficulty in adequately preventing and treating infectious diseases from such pathogens, has 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 has, in fact, shown itself capable of eliminating endemic transmission of diseases such as poliovirus, measles and rubella from the United States, and also 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 their immunogenicity and effectiveness (40). Highly successful conjugate vaccines targeting the capsular polysaccharides of *Streptococcus pneumonia* (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 human trials have been made for polysaccharide-conjugate vaccines for *Salmonella enterica* serovar typhi (20), group B streptococcus (4) and *Escherichia coli* O157 (1).

A promising target for vaccine development is a surface polysaccharide produced by a broad range of common pathogens designated poly-N-acetyl-glucosamine (PNAG), a β (1→6)-linked polymer of N-acetyl-d-glucosamine (GlcNAc) (22) with some proportion of the amino groups lacking acetate substituents (GlcNH$_2$). The basic chemical properties of PNAG were described by Mack et al. (21) where the material was referred to as the polysaccharide intercellular adhesin. Among important bacterial pathogens, PNAG is known to be produced by...
Staphylococcus aureus and Staphylococcus epidermidis (23, 25, 26), Escherichia coli (13, 42), Bordetella pertussis and B. parapertussis (29, 36), Aggregatibacter actinomycetemcomitans (15), Acinetobacter spp. (8) and Yersinia pestis (10, 12). By genetic homology, 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 only be engendered 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 non-opsonic, non-protective antibodies whereas antibodies to the core or backbone epitopes are superior in these properties, likely due to enhanced deposition of opsonically-active fragments of the third component of complement, C3b (16).

While highly, but not completely, dPNAG conjugate vaccines appear effective in animal studies at providing protective immunity, the lack of a defined chemical composition of this material, and the need to produce it by chemical deacetylation of highly-acetylated PNAG, resulting in variability in the final composition, limits the conclusions that can be drawn about optimal vaccine formulation. Native PNAG (>90% acetylated) has a certain amount of deacetylated 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 amounts of GlcNH₂ units and their spacing will need to be determined, which will
not be possible by chemical deacetylation which would randomly change GlcNAc units to GlcNH$_2$ units.

To define more precisely the immune response elicited to different epitopes on the PNAG molecule, oligoglucosamines containing either 5 or 9 fully acetylated (5GlcNAc and 9GlcNAc) or fully non-acetylated (5GlcNH$_2$ and 9GlcNH$_2$) monosaccharides were conjugated to a protein carrier (tetanus toxoid) and used to immunize mice and rabbits. The fully acetylated oligosaccharides elicited high titers of non-opsonic antibodies in mice, whereas the fully non-acetylated oligosaccharides elicited highly active opsonic antibody in mice and rabbits, with the antibody from the latter species showing excellent passive protective efficacy against *S. aureus* skin infections and *E. coli* peritonitis.

**Materials and Methods**

**Bacterial strains used.** *S. aureus* strains used were capsular polysaccharide (CP) 8 strain MN8 (18), CP5 strain Newman (3), nontyable (NT) USA 300 MRSA strains LAC (27) and SF8300 (9) and an isogenic set of strains derived from CP5 strain Reynolds wherein two genetically engineered derivatives were produced to either express no CP antigen or the CP8 antigen in place of the native CP5 antigen (43). These latter three strains were kindly provided by Dr. Jean Lee, Boston, MA. *S. aureus* strains MN8 and Newman lacking the ica locus for biosynthesis of PNAG (ica::tet) have been described previously (19). Clinical UTI isolates of *E. coli* producing PNAG (strains J and P) and a strain unable to synthesize PNAG (Strain H) have been described (7).

**Synthesis of oligosaccharide conjugate vaccine.** Production of the thiol-derivatized 5 or 9GlcNH$_2$ and 5 or 9GlcNAc oligosaccharides is graphically depicted in Fig. 1. The supplemental file (which contains the same figure as supplemental Fig. 1) also contains additional information.
experimental details and NMR spectral data to confirm the structures produced at each synthetic step. Amino groups in the spacers of the oligosaccharides (28) were selectively deprotected and acylated using linker reagents 3 and 4 (Fig. 1) to give corresponding pentasaccharide 5 and nonasaccharide 6. Introduction of the linkers was confirmed by NMR and mass-spectral data (supplementary material). After total deprotection using hydrazine hydrate in boiling ethanol, oligoglucosamine ligands with free amino groups (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 rate of oxidation is different for these two compounds, we considered ligands 7 and 9 as disulfides and used suitable reducing agents for reliable exposure of 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 (see Figure 1; 1.5 mg in 100 µl of 0.1 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 8.0) were treated with washed Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) disulfide reducing gel (200 µl of a 50% slurry in water). After incubation on a rotor rack at 22-24°C for 45 min, SH-derivatized oligosaccharides were separated from the gel by centrifugation and the immobilized TCEP was washed with the same pH 8.0 buffer (3 x 100 µl) and supernates combined.

The fully acetylated 5- and 9GlcNAc oligomers terminated with an S-acetyl group (Structures 5 and 10 in Figure 1) were dissolved in 200 µl of a 7% aqueous ammonia solution (2.1 mg of the GlcNAc oligomers) and kept at 22-24°C for 1 hour and then lyophilized.
Lyophilized oligosaccharide was immediately dissolved in 400 µl of 0.1 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 8.0.

To activate the carrier protein, TT (4 mg) was diluted with 400 µl of 0.1 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2 and a solution of N-hydroxysuccinimidyld-3-(bromoacetamido) propionate (SBAP); 2.6 mg in 80 µl of DMSO) added and incubated for 2 h at 22-24°C. Unreacted SBAP was removed using a PD-10 column in 0.1 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 8.0 and the eluate concentrated to 400 µl. The SH-activated oligosaccharides were immediately combined with modified TT protein (400 µl in pH 8.0 buffer), stirred 18-24 h at 22-24°C, then the conjugate separated from uncoupled components by gel filtration on 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 the oligosaccharide content using a hexosamine assay (37) with the corresponding free oligosaccharide used as a standard. Protein content was 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 (IACUC). Mice were immunized sc with three doses of 0.5, 2.5 or 10 µg (carbohydrate content) once a week for 3 weeks with the 5- or-9-mer GlcNAc-TT or GlcNH₂-TT vaccines. Two groups received a non-conjugated mixture of either 5GlcNAc or 5GlcNH₂ plus tetanus toxoid sc Sera were obtained prior to injection then weekly for 3 weeks after the last immunization. Rabbits were immunized subcutaneously (sc) with 10 µg (carbohydrate content) of the 9GlcNH₂-TT conjugate twice, one week apart, with an equivalent volume of Specol adjuvant. On the third week, rabbits were
immunized three times on alternate days with 10 µg (carbohydrate content) of the conjugate given intravenously in saline. After the last immunization, blood was taken two weeks after the final immunization (bleed 1) and again one month later (bleed 2).

**Antibody analysis.** Analysis of the antibody binding by ELISA to native PNAG or dPNAG antigens, prepared as described previously (23), and in an opsonic killing assay, used previously published methods (16, 23). Determination of affinity associations of the rabbit antibody to the 11GlcNH₂ and 11 GlcNAc oligosaccharides (11) as well as native PNAG and dPNAG prepared from the native PNAG (~20% acetylation) was analyzed by surface plasmon resonance (SPR) using a BIAcoreTM 3000 (BIAcore AB, Uppsala, Sweden). IgG was purified from the immune serum raised to 9GlcNH₂-TT conjugate vaccine using Protein G immobilized on agarose beads, then the IgG bound to a CM-5 sensor chip utilizing an amine-coupling kit (BIAcore AB) according to the manufacturer’s instructions. 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 Regierungspräsidium Freiburg for the University Hospital, Freiburg. Abscess formation was induced in mice by sc infection with *S. aureus* and Cytodex 1 microcarrier beads, 131-220 µm (Sigma) as described (14). *S. aureus* strains were grown in 1 L of TSB 18-24 h at 37°C with shaking at 200 rpm, cultures centrifuged and bacterial cells suspended in 10 ml of 0.9% NaCl. Aliquots were rapidly frozen and stored at -80°C. For challenge, a vial was defrosted then diluted in TSB and mixed with an equal volume of a sterile slurry of the dextran beads. Twenty-four h prior to infection, mice were injected IP with 200 µl of normal or immune rabbit serum. One-hundred µl of the

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challenge was injected into both flanks of a mouse and after 72 h the animals euthanized and the
abscesses removed, dissected, homogenized and diluted in TSB for bacterial enumeration.

The peritonitis infection model using *E. coli* was approved by the Harvard Medical Area
IACUC and followed the previously described protocol. *E. coli* was grown 18-24 h in TSB plus
1% glucose, washed, resuspended to $5 \times 10^8$ cfu/ml in PBS. An inoculum of 0.2 ml (1 $\times 10^8$ cfu
*E. coli*) was injected IP Twenty-four h before and four h after infection mice were given 0.4 ml
of either normal rabbit serum or serum with antibody raised to 9GlcNH$_2$-TT. Moribund mice
were sacrificed and counted as dead for purposes of these experiments. Spleens from
euthanized mice were recovered and cultured on MacConkey agar to ascertain that the *E. coli*
had disseminated from the initial site of infection.

**Statistical analysis.** Statistical analyses were performed using the Prism software. The Mann-
Whitney non-parametric U test for two sample comparisons was used to analyze paired data.
Non-parametric ANOVA was used for multi-group comparisons and the Dunn Procedure for
post-hoc pair-wise comparisons.

**RESULTS**

**Characterization of the oligoglucomosamine conjugate vaccines.** The hexosamine and protein
content of the conjugate vaccines is shown in Fig. 2. Conjugated oligosaccharides were readily
separated from non-conjugated 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 TT were too small to reliably separate by
chromatography.
Immune response of mice to oligoglucosamine-protein conjugated vaccines. C3H/HeN mice were immunized with the conjugate vaccines using the schedule and doses shown in Table 1. Mice immunized with 10 µg doses, based on carbohydrate content, of either the 5GlcNH$_2$-TT or 9GlcNH$_2$-TT conjugates made robust IgG responses that bound to both native PNAG and dPNAG (Fig. 3 left and middle panels). Lower doses of the GlcNH$_2$-TT vaccines gave lesser immune responses. Responses peaked 3 weeks after the final dose. Mice immunized with the 5- or 9-mer GlcNAc-TT conjugates made an excellent response to the native PNAG molecule (Fig. 3, right panel), and responses were already optimal 1 week after the last immunizing dose. However, no binding of antibody to dPNAG (not shown) was detected in the sera of mice immunized with either of the GlcNAc-TT conjugates. No immune response to either PNAG or dPNAG was detected in sera of mice immunized with a mix of TT and either the 9GlcNH$_2$ or the 9GlcNAc oligosaccharides (not shown).

When mouse sera were tested for opsonic killing activity in the presence of human PMN and rabbit complement (C‘), the animals immunized with both of the GlcNH$_2$-TT conjugates clearly had opsonically-active antibody that mediated killing of S. aureus CP8 strain MN8 (Fig. 4A). The antibody raised to both 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 to both GlcNH$_2$-TT conjugates were again opsonically active (Fig. 4B). When either 11GlcNH$_2$ or 11GlcNAc oligosaccharides were included in the opsonic assay with sera raised to either 5GlcNH$_2$-TT or 9GlcNH$_2$-TT conjugates, the non-acetylated oligomer strongly inhibited opsonic killing whereas the fully acetylated oligomer did not (Fig. 4C). Overall, the synthetic oligomers showed that non-acetylated glycoforms elicited the best opsonic
killing activity with specificity for epitopes on the homologous immunizing oligosaccharide
to the desired opsonic killing activity in mice, we used the 9GlcNH$_2$-TT conjugate to
immunize rabbits and test binding, opsonic killing and protective activities of antibodies.
Antibody obtained 2 and 6 weeks after the last injection had high titers to native PNAG and
dPNAG isolated from S. aureus, as well as to the immunizing 9GlcNH$_2$ oligosaccharide, but
failed to bind to the fully acetylated 9GlcNAc oligosaccharide (Fig. 5). 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 post-immunization rabbit antisera mediated dose-dependent opsonic
killing of a variety of S. aureus strains (Fig. 5b), with different capsular polysaccharide (CP)
types as well as to two USA 300 MRSA strains of S. aureus 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 lacking the pga genes encoding the
biosynthetic enzymes for PNAG in E. coli (Fig. 5c) (7).
Surface plasmon resonance analysis to determine the Ka and Kd of the IgG antibodies
raised to 9GlcNH$_2$-TT showed no binding of the antibody to the fully acetylated 11GlcNAc
molecule (Fig. 6B). Binding to the 11GlcNH$_2$ oligosaccharide (Fig. 6A) indicated a Ka of 3.2 ×
$10^8$ and a Kd of $3.1 \times 10^9$. Estimates of the Ka and Kd for binding to PNAG were $1.9 \times 10^7$ and
$5.2 \times 10^8$, respectively, and for binding to dPNAG (~20% acetylated) $4.2 \times 10^{10}$ and $2.4 \times 10^{11}$,
respectively (Figs. 6C-6D). However, because of the heterogeneous nature of the polysaccharide
molecules and indications of multiple binding sites on the dPNAG molecule from the SPR

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analysis, the Ka and Kd values for the polysaccharides are, at best, approximations. There was no binding of antibody raised to 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 the bacteria subcutaneously along with small (133-220 µm) dextran beads (14), injection of 200 µl of immune sera 24 h prior to infection resulted in highly significant reductions in the bacterial cfu/abscess produced by three different infectious doses of *S. aureus* MRSA strain LAC when compared with animals given NRS (Fig. 7A). Similarly, the antiserum also significantly reduced the cfu/abscess formed by CP5 strain Newman (Fig. 7B) and CP8 strain MN8 (Fig. 7C). When tested against strains Newman and MN8 unable to make PNAG due to deletion of the ica biosynthetic locus (*ica::tet*) there was no significant (P>0.05) protective efficacy (Figs. 7B and 7C). Of note, when comparing the cfu/abscess in mice treated 24 h prior to infection with NRS and then infected with either wild-type or Δica *S. aureus* strains MN8 or Newman, the mutant strains had significantly lower cfu/abscess 72 h post infection (Fig. 7D). This outcome suggests that 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 for the antiserum’s specificity, we adsorbed either non-immune or 9GlcNH₂-TT immune sera with whole *S. aureus* cells of PNAG-producing 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 (not shown) and also retained a significant ability to reduce the cfu/abscess when compared to similarly adsorbed NRS (Fig. 8). In contrast, adsorption of the antiserum to 9GlcNH₂-TT by PNAG-producing WT strain MN8 reduced antibody levels to PNAG (not shown) and removed the protective efficacy as the
Protective efficacy against *E. coli* peritonitis. Prior results have shown that most, but not all, *E. coli* isolates from urinary tract infections (UTI) make PNAG (7). We tested the protective efficacy of antibody to 9GlcNH$_2$-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 (Table 2, UTI strains J and P) whereas all controls receiving NRS did not survive. There was no protection afforded by antibody to 9GlcNH$_2$-TT antiserum against PNAG-negative *E. coli* strain H. 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 the preponderance of natural antibody binds to highly acetylated PNAG but fails to mediate opsonic killing or protection of *S. aureus* (17). Prior animal studies comparing the immunogenicity of highly acetylated PNAG and poorly acetylated (~15%) dPNAG conjugated to a non-toxic diphtheria toxoid (DT) carrier protein confirmed that the removal of acetates facilitated induction of antibody that bound to both native PNAG and dPNAG and was 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 from the fully non-acetylated, synthetic Gening et al., synthetic PNAG vaccine, Page 13
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 these antibodies readily bind to highly N-acetylated PNAG, poorly acetylated dPNAG and the non-acetylated oligosaccharides. This is a critical finding, as the actual composition of the PNAG molecules on the bacterial surface is not precisely known and likely also varies by strain, species and growth conditions. Thus, to target PNAG with protective antibodies they need to bind to the molecule regardless of the level of acetylation.

It has been proposed that more effective vaccines to microbial pathogens could be constructed by combining multiple antigens into a single vaccine. The synthesis of GlcNH₂-oligomers with a reducing end linker containing a reactive sulfhydryl group suggests 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 among strains of Y. pestis circulating in central Asia (2), making it possible 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 might enhance the protective coverage of a plague vaccine. While no formal analysis of cost has been undertaken comparing the expense 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, importantly, will not have any other microbial contaminants in the vaccine preparations.
Synthetic carbohydrate vaccines are rarely studied due to their complexity in production but conjugate vaccines containing synthetic poly (ribosyl-ribitolphosphate) provide protective immunity against *Haemophilus 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 Mabs to the *S. flexneri* 2a O-antigen to define a pentadecasaccharide representing three biological repeating units of 5 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 more success with this technology is likely to come.

Overall our findings indicate that small sized oligomers of β (1→6)-linked D-glucosamine conjugated to a carrier protein can induce high titers of opsonic antibody that is also protective against experimental *S. aureus* skin infection and lethal peritonitis due to *E. coli*. If antibody to the non-acetylated GlcNH$_2$-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 pre-clinical evaluations of this vaccine’s protective efficacy against various PNAG-producing pathogens is 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, a component of Award...
GBP and TM-L have developed intellectual property that has been licensed for development of PNAG and dPNAG-based vaccines and have received consulting income, licensing fees and royalty income from this arrangement.
REFERENCES


Table 1. Conjugates and doses used to immunize mice.

<table>
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<tr>
<th>Mouse group</th>
<th>Conjugate</th>
<th>Dose/week</th>
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<tr>
<td>(µg/mouse)(^a)</td>
<td>(3 doses total)</td>
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<tr>
<td>1, 2 and 3</td>
<td>5GlcNH(_2)-TT</td>
<td>0.5, 2.5, 10</td>
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<tr>
<td>4, 5, and 6</td>
<td>9GlcNAc-TT</td>
<td>0.5, 2.5, 10</td>
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<tr>
<td>7</td>
<td>5GlcNH(_2) + TT(^b)</td>
<td>10</td>
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<tr>
<td>8</td>
<td>5GlcNAc + TT(^b)</td>
<td>10</td>
</tr>
<tr>
<td>9, 10 and 11</td>
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<tr>
<td>12, 13 and 14</td>
<td>9GlcNAc-TT</td>
<td>0.5, 2.5, 10</td>
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\(^a\)Based on µg hexosamine.

\(^b\)Mix of oligosaccharide and TT
Table 2. Protective efficacy of antibody to 9GlcNH₂-TT against lethal peritonitis caused by *E. coli*.

<table>
<thead>
<tr>
<th>Challenge</th>
<th>Number of survivors</th>
<th>P value</th>
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<tr>
<td>E. coli strain</td>
<td>out of total mice</td>
<td>(Fisher’s exact test)</td>
</tr>
<tr>
<td></td>
<td>Anti-9GlcNH₂-TT</td>
<td>NRS&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>J (PNAG&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>8/8</td>
<td>0/8</td>
</tr>
<tr>
<td>P (PNAG&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>8/8</td>
<td>0/8</td>
</tr>
<tr>
<td>H (PNAG&lt;sup&gt;-&lt;/sup&gt;)</td>
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<td>0/8</td>
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<sup>a</sup> NRS: normal rabbit serum
FIGURES AND FIGURE LEGENDS.

FIG 1. Introduction of thiol linkers into the carbohydrate ligands. **Bold-faced** numbers indicate structures referred to in materials and methods section describing oligosaccharide synthesis and in figure 2.
FIG. 2. General scheme of conjugation and the composition of glycoconjugates used for immunization. **Bold faced** numbers 7, 8, 9 and 10 refer to structures with same number depicted in figure 1.

<table>
<thead>
<tr>
<th>Conjugate name</th>
<th>Average number of carbohydrate ligands (x)</th>
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<tbody>
<tr>
<td>5GlcNH₂-TT</td>
<td>100</td>
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<tr>
<td>5GlcNAc-TT</td>
<td>62</td>
</tr>
<tr>
<td>9GlcNH₂-TT</td>
<td>74</td>
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<tr>
<td>9GlcNAc-TT</td>
<td>71</td>
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FIG. 3. Immune response of mice immunized with GlcNH$_2$-TT or GlcNAc-TT conjugate vaccines. (a) ELISA titers to PNAG. (b) ELISA titers to dPNAG. Sera were obtained from mice immunized with indicated amount of carbohydrate equivalent of conjugates 5GlcNH$_2$-TT, 9GlcNH$_2$-TT, 5GlcNAc-TT or 9GlcNAc-TT. Bars represent means.
**FIG. 4.** Opsonic killing activity in mouse sera raised to the oligoglucosamine-TT conjugates. (A) Killing of *S. aureus* strain MN8 by different serum dilutions. Positive control serum (open bar) only tested at dilutions 1:10 and 1:80. (B) Opsonic killing of *S. aureus* strains Newman and LAC (USA 300) by a 1:10 dilution of mouse serum raised to indicated GlcNH$_2$-TT conjugate vaccine. (C) Inhibition of opsonic killing mediated by 1:20 dilution of mouse antisera to 9GlcNH$_2$-TT conjugate by synthetic oligosaccharides 11GlcNH$_2$ and 11GlcNAc. HI C’=heat-inactivated complement. Bars represent means.
**FIG. 5.** Immunologic activity of rabbit antiserum to 9GlcNH₂-TT conjugate vaccine. (A) ELISA titers to PNAG, dPNAG, 9GlcNH₂ and 9GlcNAc of antibodies obtained 2 and 6 weeks after 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* J and *E. coli* P) but not PNAG-negative (*E. coli* H) *E. coli* strains by rabbit antisera to 9GlcNH₂-TT obtained 6 weeks after the last immunization. Activity of Pre-immune serum subtracted out. C’=complement. Bars represent means.
FIG. 6. Analysis by Surface Plasmon Resonance of the binding of the indicated antigen at the indicated nanomolar (nm) concentration to rabbit IgG isolated by protein G affinity chromatography from an antiserum raised to 9GlcNH₂-TT conjugate vaccine.
FIG. 7. Protective efficacy of rabbit antibody raised to 9GlcNH₂-TT conjugate vaccine against skin abscess formation by *S. aureus*. Antisera (200 µl) were administered IP 24 h prior to injection of skin with indicated *S. aureus* strain mixed with 1 µm dextran beads. Points indicate cfu/individual abscess, lines the group median, *P* values by Mann-Whitney U test. (A) Results following challenge with three doses (indicated on X-axis) of *S. aureus* LAC (nontypable, USA 300 strain). (B) Results following challenge with wild type of *S. aureus* Newman (CP5) in comparison with lack of efficacy of antibody against *S. aureus* Newman ica::tet unable to synthesize PNAG. (C) The same comparison for *S. aureus* MN8 (CP8). (D) In mice given NRS, wild-type (WT) *S. aureus* MN8 and Newman produce more CFU/abscess than do their corresponding ∆ica mutants.
FIG. 8. Adsorption of antiserum to 9GlcNH$_2$-TT conjugate vaccine with PNAG-producing S. aureus removes protective efficacy in skin infection model. Rabbit antiserum raised to 9GlcNH$_2$-TT conjugate was adsorbed (Ads) with either ica::tet PNAG-negative S. aureus strain MN8 (MN8 ica::tet) or PNAG-producing wild-type strain MN8, then injected into mice that were subsequently infected with S. aureus MN8. The CFU/abscess at 72 h post-infection was then determined. Points indicate individual counts from an abscess, horizontal lines indicate group median. $P$ values: Kruskal-Wallis non-parametric ANOVA, pair-wise comparisons by the Dunn procedure: $P<0.05$ comparing antiserum to 9GlcNH$_2$-TT adsorbed with S. aureus strain MN8 ica::tet (Δica) with other three groups.
Tetanus Toxoid

\[ \text{TT} \]

\[ \text{TT-modified} \]

\[ \text{Carbohydrate Ligand} \]

\[ \text{AcS} \]

\[ \text{Carbohydrate Ligand} \]

\[ \text{8 or 10} \]

\[ 7\% \text{aq. NH}_3 \]

\[ \text{SBAP pH 7.2} \]

\[ \text{TT} \]

\[ \text{Carbohydrate Ligand} \]

\[ \text{TT-modified} \]

\[ \text{Carbohydrate Ligand} \]

\[ \text{Conjugate} \]

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