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To cite this article: David Skurnik, Colette Cywes-Bentley & Gerald B. Pier (2016): The exceptionally broad-based potential of active and passive vaccination targeting the conserved microbial surface polysaccharide PNAG, Expert Review of Vaccines, DOI: 10.1586/14760584.2016.1159135

To link to this article:  http://dx.doi.org/10.1586/14760584.2016.1159135
REVIEW

The exceptionally broad-based potential of active and passive vaccination targeting the conserved microbial surface polysaccharide PNAG

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ABSTRACT

A challenging component of vaccine development is the large serologic diversity of protective antigens. Remarkably, there is a conserved surface/capsular polysaccharide, one of the most effective vaccine targets, expressed by a large number of bacterial, fungal and eukaryotic pathogens: poly-N-acetyl glucosamine (PNAG). Natural antibodies to PNAG are poorly effective at mediating in vitro microbial killing or in vivo protection. Removing most of the acetate substituents to produce a deacetylated glycoform, or using synthetic oligosaccharides of poly-β-1–6-linked glucosamine conjugated to carrier proteins, results in vaccines that elicit high levels of broad-based immunity. A fully human monoclonal antibody is highly active in laboratory and preclinical studies and has been successfully tested in a phase-I setting. Both the synthetic oligosaccharide conjugate vaccine and MAb will be further tested in humans starting in 2016; but, even if effective against only a fraction of the PNAG-producing pathogens, a major advance in vaccine-preventable diseases will occur.

Vaccine potential of PNAG

Vaccines are the most exciting and effective therapeutics for human and animal diseases. They were called by the Centers for Disease Control and Infection the most important medical advance of the twentieth century [1], as they are exceedingly successful in preventing infections. Throughout the history of vaccine development, the largest challenge has been the serologic diversity of many, if not most, of the protective antigens, requiring either a large compendium of vaccine components for broad-based coverage (Streptococcus pneumoniae) [2], finding a means to deal with a continually evolving target antigen (influenza virus and human immunodeficiency virus) [3,4], or trying to address high in vivo antigenic shifts and drifts (Plasmodium spp.) [5]. Often vaccinologists must turn to finding conserved, but frequently suboptimal, antigens, or epitopes as vaccines. Thus, a major paradigm underlying the success of many infectious agents is the utilization of antigenic variation to avoid host immunity and thus retain their virulence. Overall, it would seem that a conserved antigen, readily accessible to major cellular and humoral immune effectors, would be selected against by evolutionary forces that promote pathogen success. However, over the past 20 years, it has become apparent that such a vaccine target may exist for many non-viral pathogens, and this vaccine might be developed for use in a single formulation to induce humoral immunity to the most common microbial pathogens of humans and animals, encompassing most Gram-negative and Gram-positive bacteria, including tuberculosis, as well as fungal pathogens such as Candida albicans and even important protozoan pathogens such as those causing malaria [6]. The target is poly-N-acetyl glucosamine (PNAG), a polymer of β-(1–6)-linked N-acetylglucosamine residues with a relatively small amount of deacetylation occurring on 5–15% of the monosaccharide residues (Figure 1).

To thwart host acquired immunity from being effective at preventing serious infection and disease against the collection of PNAG-producing pathogens, natural antibodies to this polysaccharide, found in most human and animal sera, are mostly ineffective at mediating microbial killing and protective immunity. Thus, these pathogens are unaffected by the native antibody response to PNAG. Fortuitously, it was discovered that by removing the acetates to produce a mostly polymeric β-1–6-linked N-glucosamine, or use of synthetic β-1–6-linked glucosamine (i.e. non-acetylated) oligosaccharides in conjugate vaccines [7–10]; antibodies could be induced that not only bound to native, acetylated PNAG but also deposited complement onto microbial surfaces, mediated in vitro killing and showed protective efficacy against a large variety of PNAG-producing pathogens in preclinical studies [6,7,9]. We now stand on the brink of determining in humans and economically important and companion animals whether immunity to PNAG will provide a massive increase in vaccine-preventable diseases, be efficacious against only some PNAG-producing microbes, or reveal unexpected consequences that could make safety considerations and side effects outweigh the benefits of a PNAG vaccine. While animal studies to date, including some ongoing studies in farm animals, are highly encouraging that the benefits will be high from a PNAG vaccine, these tests are still in the early stages. Importantly, the evaluation of the vaccine has been initiated in farm animals and will be tested in humans in 2016 along with a fully human IgG1 monoclonal antibody (MAb) to PNAG that has already undergone phase I testing [11], with phase II testing also...
scheduled for commencement in 2016. Those of us working on developing vaccines and MAbs to PNAG are cautiously optimistic about our chances to have a major impact on human and animal health, but also fully cognizant of the challenges, roadblocks, and potential of unanticipated consequences that confront the clinical development of all drugs.

Biology of PNAG

From PS/A to PIA to SAA to PNAG

Some of the properties of what eventually turned out to be PNAG were initially described as the capsular polysaccharide/adhesin (PS/A) of *Staphylococcus epidermidis*, although a definitive chemical composition and structure was not determined in these studies [12–14]. Mack et al. [13] first described the chemical properties of this surface polysaccharide and named it the polysaccharide intercellular adhesin (PIA) and attributed to PIA the property of mediating intercellular adherence of coagulase-negative staphylococci. McKenney et al. [14] found that PS/A and PIA were the same material and then reported in 1999 that the polymer was made by *S. aureus* [15], which was also reported by Cramton et al. [16]. Another variant of the PNAG polymer containing more than 70% glucosamine residues was described as the slime-associated antigen (SAA) [17]. It is now clear that all four of these entities (PNAG, PS/A, PIA, SAA) are chemically related and nearly identical [18–20].

Functional properties of PNAG

PNAG plays a major role in biofilm formation by different microbial species (see Ref. [21] for a review). In 2004, experiments reported by Romeo and colleagues designed to identify components of the *Escherichia coli* biofilm led to the discovery that many strains of this organism produce PNAG [22]. The locus in *E. coli* was named pga for polyglucosamine and bioinformatic investigations indicated a broad range of Gram-negative bacteria had the pga locus, implicating PNAG synthesis as a common factor among these organisms. Further investigations confirmed PNAG was a component of biofilms made by numerous Gram-negative bacterial species. In *Acinetobacter baumannii*, deletion of the pga locus led to loss of the biofilm phenotype, which was restored by complementation [23]. Similar findings were found with *Burkholderia cepacia* complex (BCC) [24], *Klebsiella pneumoniae* [25], and *Bordetella pertussis* [26]. Recently, PNAG's role in biofilm formation was reported in *Bacillus subtilis* [27], the first Gram-positive bacillus found to produce PNAG.

PNAG has also been found to be an important virulence factor for several pathogens. In three different strains of *S. aureus*, Mn8, Newman, and NCTC 10833 [28], loss of PNAG resulted in significantly reduced abilities to maintain bacterial levels in blood following intravenous (IV) or intraperitoneal (IP) injection in mice, or to spread systemically to the kidneys or induce a moribund/lethal state following IP infection. In *E. coli*, PNAG-deficient strains exhibited reduced fitness in the urinary bladder, kidneys, and spleen compared to wild-type strains in murine models of ascending UTI and systemic infection, respectively [29]. In *K. pneumoniae*, PNAG-negative strains are less able to colonize the GI tract and to disseminate, and are lethal to significantly fewer mice after IP infection [25].

As a major component of the bacterial biofilm, PNAG has also been reported to help bacteria evade the immune system, and particularly opsonophagocytic killing (OPK) [30], presumably by either preventing complement opsonins from binding to the bacterial cell surface or complement receptors on phagocytes from binding deposited opsonins, which are needed for efficient opsonic killing.

PNAG has also been reported to protect bacteria against environmental stresses. For instance, *S. aureus*, *E. coli*, and *K. pneumoniae* producing PNAG were all better able to survive exposure to ethanol than isogenic PNAG-deficient mutants, suggesting PNAG serves as a type of microbial armor regulating interactions of planktonic cells with the external environment [6].

Genetics and biosynthesis of PNAG

The ica and pga loci

The molecular basis for PNAG production was initially discovered in *S. epidermidis* [31]. In 1996, Heilmann et al. [31]

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**Figure 1. Structure of PNAG.**
demonstrated that each one of three genes in a locus named icaABC were necessary for the production of PNAG. An ica operon mutant lost the ability to produce PNAG, which was restored by complementation with a plasmid carrying the ica locus. In addition, PNAG production was lost when any of the three genes were individually inactivated. In 1998, a fourth gene (icaD), encoded between icaA and icaB was identified by Gerke et al. [32] who also reported that the IcaA and IcaD proteins were located in the cytoplasmic membrane. Both exhibited N-acetylglucosaminyltransferase activity, in which IcaA represented a catalytic enzyme that required IcaD for full activity.

In 2004, Wang et al. [22] used random transposon (Tn) mutagenesis to identify novel genes required for biofilm development in E. coli. They found 17 Tn-insertions clustered in a small portion of the chromosome that resulted in E. coli mutants that were producing significantly less biofilm (∼10-fold when compared to the parent strain). The locus, initially annotated as ycdSRQP, was renamed as pgaABCD. Each gene of the pga locus was necessary to produce a surface polysaccharide, whose chemical structure was determined by nuclear magnetic resonance analyses to be PNAG. From this discovery, homologs of the pga locus were found in numerous Gram-negative bacterial species [6,22–24,26,33,34]. Interestingly, the introduction of the pga locus into a PNAG-negative bacteria can lead to the production of PNAG [27]. A schematic depiction of the biosynthesis of PNAG by proteins encoded by genes in the pga and ica loci is given in Figure 2, taken from Ref. [35].

The hms locus of Yesinia pestis and eps locus of B. subtilis

PNAG has also been detected on the surface of bacterial species that have somewhat divergent genetic loci encoding PNAG biosynthetic enzymes and regulators. In Yesinia pestis, three operons, hmsHFRS, hmsT, and hmsP, are responsible for the production of biofilms [33]. PNAG has been detected in biofilms of Y. pestis, synthesized by proteins encoded within the hmsHFRS locus, and production is controlled by the diguanylate cyclase HmsT and the EAL domain-containing phosphodiesterase, HmsP. These act as positive- and negative regulators, respectively, of the transcription of the hmsHFRS locus. Amino acid sequence analysis showed that HmsR and HmsF are homologues of the S. epidermidis β-1,6-GlcNAc glycosyltransferase IcaA and deacetylase IcaB, respectively, and all four HmsHFRS proteins have 50–83% similarity to products encoded by pgaABCD.

PNAG was also detected in biofilms produced by B. subtilis [27]. In these bacteria, four genes encoding the proteins for PNAG synthesis, epsHIJK, were found in a cluster within the 15
gene eps locus encompassing epsA-epsO. Cloning each of the B. subtilis epsH-K genes into E. coli with individual in-frame deletions in each of the four PNAG biosynthetic genes, pgaA-D, respectively, restored PNAG production in E. coli. Cloning the entire B. subtilis epsHIJK locus into pga-deleted E. coli, K. pneumoniae, or alginate-negative Pseudomonas aeruginosa also restored or conferred PNAG production [27]. In Y. pestis and B. subtilis, and about seven other bacterial species, when PNAG was extracted and analyzed, it was found to be a β-1→6-linked poly-N-acetylglucosamine molecule, with only small variations in the levels of amino-group acetylation (95–100%) and variation in the amount of O-linked acetates and succinates [22,36,37].

Detection of PNAG expression by a broad range of microbial pathogens

To detect the presence of PNAG on microbial cells, immunofluorescence microscopy using an antigen-specific MAb to PNAG, designated F598, is routinely used [38]. The specificity of the MAb has been confirmed in studies wherein binding is strongly detected using wild-type E. coli, S. aureus, and A. baumannii cells, but not to these bacteria deleted for the pga or ica loci [6,23,39]. Binding is restored when an intact pga or ica locus is placed onto a plasmid or back into the chromosome. Another test uses the PNAG-degrading enzyme dispersin B that specifically hydrolyzes β-(1→6)-linked N-acetylglucosamine residues [40,41] to degrade the antigen and show concomitant loss of MAb F598 binding. Control treatment is with the β-1→4-linked N-acetylglucosamine hydrolyzing chitinase. Chitinase-resistant but dispersin B-sensitive binding of MAb F598 is highly specific to PNAG (Figure 3). This approach is particularly useful for identifying PNAG expression when encoding genes have not been fully identified. At the moment, PNAG production has been identified in over 30 microbial organisms (Table 1) including fungal (C. albicans) and protozoan parasites (Plasmodium falciparum) [6], using chemical, genetic and/or immunologic analyses. For a number of these, the protective efficacy of antibody to PNAG in mouse models of infection has confirmed the antigen is expressed in vivo and a vaccine target [6]. Extensive work is ongoing to determine the precise genetic and molecular basis for PNAG production in all these species.

In vivo expression of PNAG

Previous studies have reported PNAG expression in the spu-
tum and lung of CF patients infected with S. aureus [15], with PNAG detected by fluorescent microscopy at the surface of 80–90% of the bacterial cells. PNAG was also found on the surface of microbial cells in vivo in the lungs of patients infected by BCC [24] or Mycobacterium tuberculosis [6]. In children, in vivo expression of PNAG was found in the middle
ear effusions from *S. pneumoniae* or nontypable *Hemophilus influenzae* otitis media samples [6].

In animals, *in vivo* expression of PNAG was found in nasopharyngeal fluid of chinchillas experimentally infected by *S. pneumoniae* serogroup 19A, in the gastrointestinal tract of a mouse experimentally infected by *C. rodentium*, and in ocular tissues from mice with *C. albicans* keratitis [6]. PNAG is also expressed by major symbiont bacteria found in the human microbiota: the GI tract (*E. coli* among others), the skin (*S. epidermidis*), and the oral flora (*Aggregatibacter actinomycetemcomitans*). The extensive *in vivo* expression of PNAG raised the question of whether natural antibodies to PNAG were present in healthy individuals and whether these antibodies were protective.

### Naturally occurring antibodies to PNAG

Interestingly, in several studies, high levels of antibodies to PNAG were detected in healthy humans [8,38,42,43] by enzyme-linked immunosorbent assay (ELISA). Notably, these antibodies do not appear to be functional or protective. Natural antibodies to PNAG are not able to mediate *in vitro* bactericidal or OPK [8] and they do not protect animals against PNAG producing pathogenic bacteria such as *S. aureus* [42]. The natural antibodies predominantly require that the acetate substituents on PNAG be present in order to bind to the antigen, but the result is an inability to mediate complement activation and protective immunity [8,9,42]. Structural studies of PNAG indicate that in its preferred glycoform conformation the acetates are on the polymer’s surface, sticking out into the surrounding medium, and this forms the basis for the immunodominance of the acetate-dependent epitopes [44]. Apparently, then, the Fc regions of the antibodies bound to the acetate-containing epitopes are not sufficiently close together to effectively bind C1q and initiate complement activation [38,42].

### Immunity to PNAG and capsular polysaccharides in *S. aureus* infections

In contrast to most normal human sera, in some infected patients functional antibodies to PNAG could be detected [8], especially opsonizing antibodies in patients with *S. aureus* bacteremia [43]. However, most patients with non-bloodstream, acute *S. aureus* infections, such as in the skin and lung, failed to make an opsonizing antibody response to PNAG. Thus, either a more intense, or longer term, infection was required to elicit opsonic antibodies to PNAG in the context of *S. aureus* infection. Notably, the fact that humans could make such antibodies supported other data that altered glycoforms of PNAG could induce opsonic, protective antibody [9,39] indicating it would be feasible to develop an effective passive or active immunotherapy targeting PNAG.

With the discovery of extensive production of PNAG by clinical isolates of *S. aureus* it became very attractive to consider a multivalent vaccine incorporating this antigen with the *S. aureus* capsular polysaccharides (CP), based on analogies with successful capsular vaccines to other bacterial pathogens, including *S. pneumoniae*, *Neisseria meningitidis*, *H. influenzae* type b, and *Salmonella enterica* serovar Typhi [2]. *S. aureus* makes two CP antigens, types 5 and 8, expressed by 70–85% of clinical isolates and are considered to be excellent candidate vaccine antigens [45]. Unfortunately, two clinical trials for preventing bacteremia in hemodialysis patients using CP antigens conjugated to a carrier protein as a vaccine both failed to meet their primary end points of reduced bacteremia [46,47]. Notably, in the first trial [46], there was 57% protective efficacy (*P* = 0.02) after 40 weeks, as determined by a post hoc analysis; but at the predetermined 54-week endpoint efficacy was only 26% (*P* = 0.23). Thus, a multivalent PNAG/CP5/CP8 vaccine seemed like a reasonable candidate to augment the limited efficacy of the bivalent CP vaccine.

However, when testing of antisera containing antibodies to these three components was started, instead of observing the expected additive or synergist effects, what was actually found were potent and mutually inhibitory effects on opsonic killing using *in vitro* assays and mouse models of bacteremia and skin infections [43]. Electron microscopy, isothermal calorimetry, and surface plasmon resonance experiments indicated that antibodies to CP and PNAG bound together via an apparent charge-dependent, idiotype–anti-idiotype interaction. In the sera of patients recovering from *S. aureus*, bacteremia antibody titer increases to both PNAG and CP antigens were found, but even in these sera there was a notable lack of *in vitro* and *in vivo* protective efficacy [42,43]. Consistent with the laboratory-based findings of potent inhibitory effects between
immunization-induced antibodies to PNAG and to S. aureus CP antigens, infection-induced antibodies to both PNAG and the S. aureus CP, which by themselves were able to mediate opsonic killing, did not do so when both antibodies were in the same serum sample. When either antibodies to PNAG or capsule were removed by absorption, the sera gained functional activity and this was again lost when the two antibodies were recombined [43]. In a follow-up study, it was reported that natural, non-opsonic antibodies to PNAG that are present in most NHS samples can also neutralize the functional activity of antibodies to S. aureus CP5 and CP8 antigens, but these natural antibodies to PNAG do not interfere with functional antibodies to PNAG [42]. These findings raised the possibility that a potential explanation for the failure of the CP-based vaccines in hemodialysis patients might have been loss of efficacy when the titers induced by CP immunization declined to the point where natural antibodies to PNAG neutralized their protective efficacy. Altogether, these findings show that the presence of natural antibodies to PNAG in both healthy and infected subjects could impact newer attempts to use the CP antigens as vaccines but also highlight the complexity of vaccine development using PNAG or CP antigens for S. aureus.

**Discovery of the means to successfully induce functional immunity to PNAG**

Before the full characterization of the chemical structure of PNAG, immunization-induced antibodies raised to what was most likely semi-purified PNAG that had undergone extraction processes for purification that likely altered its composition were reported to be opsonic in vitro and active in vivo against S. epidermidis [48,49]. Once the composition of native PNAG was known, the basis for protective efficacy in comparison to the lack of functionality of natural antibodies to PNAG could be explained.

Analysis of purified PNAG that induced opsonic killing and animal protection showed a markedly reduced level of N-acetylation in this material [9,19]. Based on this finding, two conjugate vaccines using native, highly acetylated (~95%) PNAG and the deacetylated glycoform, termed dPNAG (~15% acetylation), conjugated to a carrier protein of mutant diphtheria toxoid were synthesized. Antibodies to dPNAG-DT were found to be much more effective than antibodies to native PNAG-DT at inducing opsonic killing and reductions in bacterial levels in the blood of bacteremic mice and at protecting mice in a model of lethal S. aureus infection [9].

An additional study examining the opsonic killing activity against S. aureus in serum samples from infected CF patients found that antibodies directed at the deacetylated (backbone) epitopes on PNAG (dPNAG) were superior at eliciting in vitro opsonic killing. More precisely, it was found that when opsonic killing activity was detected in the CF patient’s sera, antibodies directed to dPNAG were more functional in phagocyte-dependent killing than antibodies directed at the acetate-dependent epitope (PNAG). This result was obtained using both whole serum and affinity-purified antibody populations directed at the epitopes under examination [8]. Of note, the presence of such antibodies correlated with a healthier overall clinical condition in spite of the presence of S. aureus in sputum cultures. However, it is a hallmark of CF that patients with S. aureus are often healthier than those lacking this organism, although the most likely explanation is that lack of S. aureus is also associated with the presence of P. aeruginosa, the pathogen clearly most responsible for clinical deterioration in CF [50,51]. Nonetheless, the finding of functional antibodies to PNAG in S. aureus-infected CF patients could account for the relative lack of pathology associated with the presence of this pathogen in the lungs of these patients.

As both the animal-derived [9] and human-derived antibodies to dPNAG [8] were better able to mediate OPK activity than antibodies to native PNAG, human monoclonal antibodies (MAbs) to PNAG and dPNAG were produced to further study this phenomena [38]. A MAb designated F598 that bound well to both native PNAG (expressed on microbial surfaces) and dPNAG (which binds the functional antibodies) had superior opsononophagocytic activity and protective activity in a mouse model of lethality induced by S. aureus than two MAbs that bound optimally to PNAG and minimally to dPNAG (MAbs F628 and F630). The better activity of antibodies to dPNAG was associated with deposition of opsonically active fragments of the first and third components of complement onto PNAG [38] (Figure 4).

As the natural antibodies to PNAG are directed against its native, highly acetylated (>90% of N-acetyl groups) form, these results probably explain the lack of protection mediated by natural antibodies: they do not bind to dPNAG or deposit C3 opsonins onto bacterial surfaces [8,38]. In contrast, dPNAG elicits antibodies with excellent engagement of the complement system leading to robust in vitro killing activity and protection against infection in experimental animal models [8,38,42]. Thus, the dPNAG glycoform is the basis for the current vaccine approaches being evaluated against PNAG-producing microbes.

**Production of synthetic oligosaccharide-protein conjugate vaccines**

A further refinement to the development of a product for active immunization was achieved by conjugating synthetic oligosaccharides composed of either non-acetylated β-(1–6)-d-glucosamine (GlcNH₂) or β-(1–6)-N-acetylg glucosamine (GlcNAc) to carrier proteins [7,10]. Oligosaccharides of either five or nine monosaccharides in length, with linkers on the reducing termini that could be activated to produce sulfhydryl groups for conjugation to bromoacetyl groups introduced onto carrier proteins, were synthesized [7]. Interestingly, synthetic S-met GlcNH₂ (5GlcNH₂) or 9GlcNH₃ conjugated to tetanus toxoid (TT) elicited mouse antibodies that mediated opsonic killing, while the antibodies that were produced in response to the acetylated oligosaccharide conjugate vaccine, 5GlcNAc- or 9GlcNAc-TT, did not mediate opsonic killing [7]. 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 [7]. Subsequent studies showed other carrier proteins could induce protective antibodies against S. aureus and E. coli when conjugated to 5GlcNH₂ or 9GlcNH₂ [10,52]. Therefore,
the synthetic 5GlcNH₂ oligosaccharide now forms the basis for the clinical development of PNAG-targeting vaccines for humans and animals.

**In vitro correlates of PNAG-mediated immunity**

Eliciting antibodies to PNAG with the proper properties to mediate microbial killing and clearance is essential for achieving protective immunity, and predicting the level and functionality of antibodies that are indicative of protective immunity is key for evaluating vaccine doses and constructs for their potential to mediate protection. A number of *in vitro* assays have been developed as functional correlates of immunoprotection.

**Antibody titer measured by ELISA**

In order to distinguish the levels of naturally occurring, usually nonprotective, antibodies to PNAG from functionally active, vaccine-elicited antibodies to dPNAG, ELISA-based assays using either purified PNAG- or dPNAG-coated plates are employed. The serum antibody titers are interpreted as the amount of PNAG or dPNAG bound antibody determined by optical density. Linear regression formulas are generated to calculate titers. While antibodies to both PNAG and dPNAG will bind native PNAG, only dPNAG-elicited antibodies will bind dPNAG, in contrast to most natural antibody to PNAG [7–9]. While a validated ELISA test or titer for either PNAG or dPNAG is not yet developed beyond those used in laboratory investigations, it is clear that protective antibodies must bind to both of these antigens, indicating that a high titer to dPNAG is a good measure of likely protective efficacy.

**Complement mediated opsonic and bactericidal killing**

For Gram-positive bacteria, the OPK assay can be used to demonstrate both antibody specificity for PNAG recognition, and functional activity, by use of neutrophil- and complement-mediated killing of opsonized pathogens. Similarly, for Gram-negative bacteria, the bactericidal assay using antibody to PNAG/dPNAG and complement demonstrates antibody specificity and functionality, requiring the complement membrane attack complex, consisting of complement components C5–C9, to be formed. These assays constitute core components of the *in vitro* evaluation of vaccine-induced antibodies to PNAG. Whether all strains of Gram-negative bacteria are killed in the absence of phagocytes is not known.
Complement deposition assays

Another means used to correlate the PNAG antibody titer with functional activity is use of a C3 deposition assay [38,42]. In the most recent iteration of this assay, ELISA plates are coated with purified PNAG and then varying concentrations of heat-inactivated (to remove endogenous complement activity) dilutions of pre- and postimmunization sera, and an active complement source, kept at a constant concentration, are added. Functionally active PNAG-specific antibodies bind to the purified PNAG on the ELISA plate and activate complement, resulting in C3 deposition. A C3-specific antibody is used to detect the amount of C3 deposited in each well by the PNAG-specific antibody. Thus far, the amount of C3 deposited has correlated well with PNAG-specific antibody titers and protection against experimental infection challenges, a finding applicable to both polyclonal and MABs [38].

More recently, it was found that protective antibodies to PNAG mediate their activity via activation of the classical complement pathway (Figure 4). PNAG itself can activate complement via the lectin pathway, as evidenced by antibody-independent C3 deposition occurring in C1-deficient serum [53]. However, this pathway is not optimal for mediating killing of many Gram-positive bacterial species [54], whereas antibody-mediated activation of the classical pathway is much more effective. Therefore, we have developed a C1q-deposition assay similar to that for C3 deposition, except an antibody specific to C1q is used to detect complement activation and binding of C1. Of note, C1q is highly conserved across species and a single antiserum to purified human C1q can be used to detect C1 deposition using sera obtained from a range of mammalian species as the complement source.

In vivo studies

Since the early 1990s, antibodies to PNAG have shown a broad-spectrum activity against numerous pathogens in laboratory animal models. Published results are summarized in Table 2 and Cwyces-Bentley et al. [6] provide experimental results with Streptococcus pyogenes, Listeria monocytogenes, S. pneumoniae, and N. meningitidis serogroup B. In addition, the human MAB to PNAG protected T-bet/RAG2-deficient mice from infectious colitis [6,55].

In vivo protection against staphylococci infections

The first report of protective activity of antibodies to PNAG (called PS/A at the time) was in a rabbit model of bacteremia using catheters colonized by coagulase-negative staphylococci inserted into the right jugular vein and attached to a subcutaneous osmotic pump [48]. In this model, antibodies to PNAG were able to reduce the number of bacteremic days by approximately 60%. This approach was then extended to a rabbit model of endocarditis [49], and both passive and active immunization targeting PNAG protected against bacteremia and endocarditis cause by S. epidermidis. The antibodies to PNAG were next tested against S. aureus in numerous studies. In the first report [15], active and passive immunization significantly reduced the colony forming units (CFU) recovered from the kidneys of mice after IV infection with different strains of S. aureus. Similarly, passive immunization significantly protected mice in a lethality model. In the ensuing 16 years, antibodies to PNAG were repetitively shown to be protective against S. aureus infections, including mouse models of bacteremia and skin abscesses as well as sheep mastitis [7–9,19,28,38,42,43,56,57].

In vivo protection against infections caused by E. coli

E. coli represents a major challenge for vaccine development, mostly due to the large serologic diversity of protective antigens such as LPS O side chains and capsules [58–60]. After the report that the conserved PNAG was also present on the surface of E. coli [22], antibodies to this antigen were tested for protection against this organism. In several studies, using a model of systemic dissemination after IP infection [7,34,39], both polyclonal and MABs to PNAG significantly protected mice against various strains of E. coli. For infections caused by E. coli colonizing the GI tract, the 9GlcNH₂ synthetic oligosaccharide conjugated to Shiga toxin 1b subunit (9GlcNH₂-Stx1b) was shown to induce rabbit antibodies that were protective against infection caused by different strains of Shiga-toxin producing E. coli [52]. Thus, in multiple in vivo mouse settings, antibodies to PNAG were effective in reducing the consequences of E. coli infections.

In vivo protection against infections caused by multi-drug resistant and pan-drug resistant Gram-negative pathogens

Antibiotic resistance is a major concern impacting treatment of illnesses caused by Gram-negative bacteria, and a vaccine or immunotherapeutic alternative is highly desirous to participate in curing these infections. Among the main pathogenic Gram-negative bacteria that are naturally multi-drug resistant (MDR) as well as capable of acquiring additional resistances to become pan-drug resistant (PDR) are A. baumannii and strains within the BCC. Studies with A. baumannii, a major cause of nosocomial infections in immunocompromised patients, showed that rabbit antisera to 9GlcNH₂-TT significantly reduced levels of A. baumannii in the lungs or blood of mice 2 or 24 h post-infection, respectively, compared to levels in control groups receiving normal rabbit sera [36]. There are three major BCC species:
Burkholderia cenocepacia, B. multivorans, and B. dolosa and they can all cause PDR infections, particularly in patients with cystic fibrosis. All three species were shown to produce PNAG both in vitro and in vivo. Antibodies to PNAG were able to kill these bacteria in opsonophagocytic assays as well as protect mice from lethal peritonitis caused by BCC strains injected either alone or in combination with MRSA, chosen as another example of a PNAG-producing pathogenic and antibiotic-resistant bacterium [24].

Carbapenems are the main drug used to treat infections caused by MDR Gram-negative bacteria in general, and MDR enterobacteriacea in particular. Unfortunately, there are now more and more Carbapenem Resistant Enterobacteria (CRE), that have emerged mainly through the acquisition of genes encoding for production of carbapenemases such as the K. pneumoniae carbapenemase or the New Delhi metallo β-lactamase [61–63]. As with the other antibiotic resistant strains, antibodies to PNAG were able to kill CRE strains in vitro and protect mice in vivo against three clinically relevant species of enterobacteriacea (E. coli, Enterobacter cloacae and K. pneumoniae), whether or not the strains produced a carbapenemase [34]. Finally, while P. aeruginosa is the only major drug-resistant pathogenic bacterium not producing PNAG naturally, antibodies to PNAG were shown to be able to protect mice infected by a PNAG producing, genetically engineered P. aeruginosa construct, suggesting that regardless of the Gram-negative bacterial species; PNAG expression is the sole determinant of the protective efficacy of antibodies to this antigen. The animal models used in these studies involved both IP and IV injections of the bacteria [34].

Killing and protection against pathogenic eukaryotes by antibodies to PNAG

PNAG has been detected at the surface of several eukaryotes, including human pathogens such as C. albicans and P. falciparum [6]. Using a model of mouse keratitis, antibodies to PNAG significantly reduced CFU counts recovered from the cornea of C57BL/6 mice infected with C. albicans and treated with the human MAB F598 to PNAG either before or after the challenge by C. albicans [6]. These antibodies also killed C. albicans in vitro using an OPK. When Plasmodium berghei was used to infect mice, which is a relevant murine model for P. falciparum human infection, antibodies to PNAG significantly reduced the mortality of C57BL/6 mice from systemic PNAG-positive P. berghei ANKA infection initiated by IP injection [6]. Thus, the potential efficacy of PNAG as a vaccine extends to a very broad range of targets.

Route to a clinical testing

Although the first papers describing protective efficacy against S. epidermidis using injections of what is now known to be PNAG-containing semi-purified antigens were published 25 to almost 30 years ago [12,49], it has taken this long to bring this technology to the point of human testing. Two products are being evaluated. One is the fully human IgG1 molecule derived by cloning variable regions from a human B cell obtained from an individual recovering from a S. aureus infection into the TCAE 6.2 vector encoding the human gammal heavy chain constant regions and lambda light chain constant region [38]. The second is a synthetic pentamer of β1–6-linked glucosamine pentamers bound together by a disulfide bond at the end of a linker, as described [7]. Commercially available, GMP-grade TT is used as the carrier protein. Trials of this vaccine, containing from 20–39 oligosaccharides per TT monomer, have commenced in economically important farm animals with some encouraging, albeit early, results indicating efficacy. As these trials are ongoing the full results are not yet available, but at least for some that are successful, it is anticipated that findings will be reported at appropriate meetings and potentially published in 2016.

Phase I clinical trial of MAb

The human IgG1 MAb was tested in 20 healthy humans in 2010 in a dose-escalating trial involving four volunteers per dose, five doses given IV in a 2-h infusion starting at 0.85 mg/kg and ending at 17.2 mg/kg [11]. There were no significant adverse reactions, but two individuals given the highest dose had recordable events, one involving a rash that developed after infusion that was resolved by use of diphenhydramine and the other an infusion-reaction resolved by slowing the rate of infusion. The half-life of the MAB was ~28 days and high titers of functional, opsonic antibody to S. aureus were maintained throughout the 50-day observation and blood sampling periods. No reports of distress that might be related to mucosal surface reactions or disruption to the normal microbiota were recorded. Overall, it appeared the MAB to PNAG is safe with an outstanding half-life and other properties, paving the way for phase II trials.

Phase I clinical trial of vaccine

The synthetic pentameric 5GlcNH2-TT has undergone about 2 years of development to scale up the synthesis of the oligosaccharide, which is produced as a dimer of two glucosamine pentamers bound together by a disulfide bond at the end of a linker, as described [7]. Commercially available, GMP-grade TT is used as the carrier protein. Trials of this vaccine, containing from 20–39 oligosaccharides per TT monomer, have commenced in economically important farm animals with some encouraging, albeit early, results indicating efficacy. As these trials are ongoing the full results are not yet available, but at least for some that are successful, it is anticipated that findings will be reported at appropriate meetings and potentially published in 2016.

Phase II clinical trials

Of note, with the broad range of pathogens producing PNAG, the challenge for phase II (and beyond) trials is finding a setting for testing both the MAb and vaccine where infection rates will be high. One obvious setting is human challenge models [64], and some that are currently available for PNAG producing pathogens include Neisseria gonorrhoeae [65], P. falciparum [66], Hemophilus ducreyi [67], and several GI pathogens [68,69]. Additional trials conducted in more traditional settings where infection rates with PNAG producing
pathogens is high might include efficacy in intensive care units, GI tract surgery, mechanically-ventilated patients or similar high-risk locations within hospitals and health-care settings. Interventional studies with both the MAb and vaccine in either newly diagnosed patients with long or difficult courses of antibiotic treatments, such as drug-resistant tuberculosis or MDR Gram-negative pathogens, or recurrent infections, such as methicillin-resistant *S. aureus* skin and soft-tissue infections or *E. coli* urinary tract infections, are also being considered. Overall the final decisions will depend on numerous factors, taking into consideration concepts such as medical need and potential impact on health, settings providing a proof of principle, economic feasibility of the intervention and any other related risk/benefit considerations.

**Expert commentary**

The potential for a single vaccine component to provide protective immunity to so many major pathogens is a clear paradigm shift in the field. In favor of realizing much of this potential is the broad-based expression of PNAG [6], indicative of an essential role in microbial biology inasmuch as so many divergent genetic and enzymatic methods to produce PNAG have evolved and survived in diverse microbial populations [22,23,27,31,33], detection of *in vivo* expression during human and animal infection [6,15], and preclinical data mostly in mice demonstrating protection based on use of the deacetylated glycoform of PNAG to induce immunity [7,9]. Furthermore, as a surface, essentially capsular, polysaccharide [6], PNAG is also available to immune effectors as are the more traditional capsules of *S. pneumoniae, H. influenzae* type B, and *N. menigitides*. Co-occurrence in the same physical space on the microbial surface with classic capsules, among our most successful vaccines, places PNAG in an excellent position to be an effective vaccine target. Many interesting questions remain related to the diversity of genes and proteins producing PNAG in so many different species, including their functions [32,70,71], how highly conserved the overall PNAG structure is, and how essential PNAG is to a given microbe’s fitness for survival. Nonetheless, the field is currently ready to see the results of the animal and human testing to ascertain if in settings of infections that are relevant to specific but different animals hosts immunity to PNAG can be effective.

On the down-side, use of the MAb in humans and the vaccine in animals is still quite limited, so overall safety considerations are still predicated on a small sample size. Long-term effects are not known, nor is the duration of immunity, and whether protective antibody isotypes will be universally induced cannot be predicted from the small sample sizes tested to date. Fortunately, there have been no indications that the natural antibodies to PNAG that most everyone possess, interfere with the vaccine-induced antibodies, but it would not be surprising if some situations arise wherein the efficacy of vaccine-induced antibodies is compromised by natural antibodies, including those to *S. aureus* CP antigens. Notably, several companies are pursuing *S. aureus* vaccines containing the CPS and CP8 antigens [72,73] and if vaccines targeting both these antigens and PNAG are found to be efficacious, this could present a difficult problem if these vaccine-induced antibodies are cross-neutralizing, as has been reported using rabbit and mouse antibodies [42,43].

**Five-year view**

At this point, the vast majority of vaccine research is centered on finding individual vaccines for every important microbe. This is the basis for not only overall vaccine success to date but is also consistent with our modern understanding of immunity and host defense in that serologically variant and organism-specific antigens maximize their pathogenic potential. In the next 5 years, extensive testing of the PNAG MAb and vaccine will ensue. During this period, clear answers as to safety will be obtained, as will a reasonable amount of efficacy data, particularly in economically important animals. Success of either reagent in human challenge models, or a clearly medically important clinical setting, will likely propel the licensing of PNAG-directed immunotherapies. Given the exceptionally broad range of PNAG-targetable microbial species, it might be anticipated there will be some failures for specific organisms or in specific tissue settings. This could be due to lack of *in vivo* PNAG expression, difficulty in getting immune effectors (antibody, complement, phagocytes, Th17 cells, act) to sites of infection (i.e. skin, urogenital tract, GI tract) or emergence of PNAG-negative variants that retain pathogenicity. Significant safety issues, mostly not anticipated at this time, could be a major barrier to development. Concerns about effects of antibody to PNAG on normal microbial flora has not been realized in any of the animal or short-term human studies conducted to date, but larger studies might find problems here. A difficult to predict consequence impacting the field might entail the success of immunity to PNAG in preventing infections with some pathogens, but possibly exacerbating infections by others, as antibody-mediated enhancement of infection is not unknown in infectious diseases, particularly with viral pathogens [74]. Overall, significant clinically relevant results in humans with the MAb and the 5GlcNH$_2$-TT vaccine and in animals with the vaccine will be obtained in the next 5 years. These likely will determine the potential success or failure of targeting PNAG with a broad-spectrum antibody and vaccine, not unlike broad-spectrum antibiotics. Optimistically, the immunotherapies will have little downside and thus provide humanity with an important new tool to control infectious diseases.

**Key issues**

- PNAG is an exceptionally broadly expressed capsular/surface antigen on most major human and animal pathogens.
- Immunity to PNAG can be engendered by use of deacetylated/non-acetylated glycoforms, particularly as components of conjugate vaccines.
- PNAG likely has an essential role in microbial biology based on its broad expression and the diversity of genetic and enzymatic proteins involved in its synthesis among different microbial species.
- Natural immunity to PNAG is common but mostly ineffective due to an inability of natural antibodies to activate complement.
Preclinical data in mice show broad-protection against bacterial, fungal, and protozoan pathogens.

Phase I trials of a fully human IgG1 MAb to PNAG showed a long half-life and overall excellent safety profile.

Human clinical trials of the MAb and a conjugate vaccine consisting of 5GlcNH₂-TT will commence, barring unforeseen difficulties, in 2016.

Evaluation of the vaccine in economically important and companion animals has commenced with results likely available in 2016.

Challenges remain in regard to long-term safety, efficacy against a range of pathogens and unanticipated consequence.

Financial and competing interests disclosure
GB Pier is an inventor of intellectual properties [human monoclonal antibody to PNAG and PNAG vaccines] that are licensed by Brigham and Women's Hospital to Alopexx Vaccine, LLC, and Alopexx Pharmaceuticals, LLC, entities in which GB Pier also holds equity. As an inventor of intellectual properties, GB Pier also has the right to receive a share of licensing-related income (royalties, fees) through Brigham and Women's Hospital from Alopexx Pharmaceuticals, LLC, and Alopexx Vaccine, LLC. GB Pier's interests were reviewed and are managed by the Brigham and Women's Hospital and Partners Healthcare in accordance with their conflict of interest policies. D. Skurnik and C. Cywes-Bentley are inventors of intellectual properties [use of human monoclonal antibody to PNAG and use of PNAG vaccines] that are licensed by Brigham and Women's Hospital to Alopexx Vaccine, LLC, and Alopexx Pharmaceuticals, LLC. As inventors of intellectual properties, C. Cywes-Bentley and D. Skurnik also have the right to receive a share of licensing-related income (royalties, fees) through Brigham and Women's Hospital from Alopexx Pharmaceuticals, LLC, and Alopexx Vaccine, LLC. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial consequence.

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References
Papers of special note have been highlighted as:
• Of interest
** Of considerable interest


** This paper describes the broad-based expression of PNAG among a range of microbes and documents the protective efficacy of polyclonal and MAb to PNAG against these pathogens. It also highlights the common in vivo expression of PNAG in infected tissues.


• This paper documents the utility of synthetic oligosaccharides as a basis for a conjugate vaccine to PNAG. It also validates the need for non-acetylated glucosamines as the PNAG glycoform needed top-induced protective immunity.


-- In 2004, Romeo and colleagues found the genes encoding the PNAG biosynthetic proteins in E. coli, leading to the identification of these genes in a large number of Gram-negative pathogens.


• This publication provided the initial identification of the genetic locus encoding proteins involved in PNAG synthesis and insight into the function of these proteins.


• This review article presents an outstanding overview of the biosynthesis of PNAG.


• This paper describes the production and characterization of MAB FS59 to PNAG, no in clinical trials in humans and also used as the reagent to detect PNAG among microbial pathogens.


• The use of Dispersin B as a tool in identifying and studying PNAG is exceptional. This paper reports on its properties and its specificity for the β-1-6-linkage between the glucosamines in PNAG.


