Acinetobacter baumannii has emerged as a highly troublesome, global pathogen. Treatment is complicated by high levels of antibiotic resistance, necessitating alternative means to prevent or treat A. baumannii infections. We evaluated an immunotherapeutic approach against A. baumannii, focusing on the surface polysaccharide poly-N-acetyl-β-(1-6)-glucosamine (PNAG). We used a synthetic oligosaccharide of 9 monosaccharide units (9Glc-NH₂) conjugated to tetanus toxoid (TT) to induce antibodies in rabbits. In the presence of complement and polymorphonuclear cells, antisera to 9Glc-NH₂-TT mediated the killing of A. baumannii S1, a high-PNAG-producing strain, but not its isogenic PNAG-negative, in-frame deletion mutant strain, S1 Δpga. Complementing the pgaABCD locus in trans in the shuttle vector pBAD18kan-orí, plasmid Δpga-c, restored the high levels of killing mediated by antibody to PNAG observed with the wild-type S1 strain. No killing was observed when normal rabbit serum (NRS) or heat-inactivated complement was used. Antiserum to 9Glc-NH₂-TT was highly opsonic against an additional four unrelated multidrug-resistant clinical isolates of A. baumannii that synthesize various levels of surface PNAG. Using two clinically relevant models of A. baumannii infection in mice, pneumonia and bacteremia, antisera to 9Glc-NH₂-TT significantly reduced levels of A. baumannii in the lungs or blood 2 and 24 h postinfection, respectively, compared to levels of control groups receiving NRS. This was true for all four A. baumannii strains tested. Overall, these results highlight the potential of PNAG as a vaccine component for active immunization or as a target for passive antibody immunotherapy.

Acinetobacter baumannii is a Gram-negative coccobacillus that recently has emerged as a major cause of health care-associated infections worldwide, and it is associated with high rates of morbidity and mortality, extended hospital stays, and significant health care expenses (2, 3, 19, 28, 35).

Among the most common types of infections caused by A. baumannii is ventilator-associated pneumonia in patients confined to hospital intensive care units (ICUs), as well as bacteremia, urinary tract infections, skin and soft-tissue infections, and bone infections (26). A. baumannii infections have frequently been reported in trauma victims (1, 21, 27), and recently a large number of infections due to A. baumannii have been reported in U.S. soldiers returning from the Iraq and Afghanistan conflicts (6, 8, 32).

Antimicrobial resistance among Acinetobacter species has been described with increasing frequency in the past decade (20), encompassing high-level resistance or multidrug resistance (MDR) to ampicillin (Amp)-sulbactam, aminoglycosides, fluoroquinolones, and carbapenems (2, 13, 15, 17, 20, 30). Moreover, the emergence of MDR A. baumannii isolates with decreased susceptibility to tigecycline and colistin, two antibiotics considered last resort against this pathogen, has been reported (5). The capacity of Acinetobacter species for extensive antimicrobial resistance may be due in part to the relatively low permeability of Acinetobacter to antibiotics (31) and its acquisition of a large number of different resistance genes acquired from routine environmental exposures (7).

We have previously reported that the surface-associated polysaccharide poly-N-acetyl-β-(1-6)-glucosamine (PNAG) was expressed by a large number of clinical isolates of A. baumannii and played a critical role in the ability of this bacterium to form biofilms in vitro (10). We now report on the potential of PNAG to serve as a target for protective immunity against A. baumannii infections. Using rabbit antibodies to a fully synthetic nonameric β-(1-6)-glucosamine oligosaccharide, 9Glc-NH₂ conjugated to the carrier protein tetanus toxoid (9Glc-NH₂-TT), we found that these antibodies mediated high levels of killing of PNAG-producing, but not PNAG-negative, A. baumannii, and we also demonstrate that these antibodies have protective efficacy in two clinically relevant murine models of A. baumannii infection, bacteremia and pneumonia.

**MATERIALS AND METHODS**

**Bacterial strains.** The bacterial strains used in this study are listed in Table 1. All strains were routinely grown in lysogeny broth (LB) or on LB agar plates, except for the strain A. baumannii S1 Δpga-c (strain S1 Δpga complemented with the pgaABCD genes), which was grown in LB broth/agar supplemented with 50 µg kanamycin/ml.

**Opsonophagocytic assay.** Polymorphonuclear cells (PMNs) were prepared from fresh human blood collected from healthy adult volunteers as described elsewhere (23) under a protocol approved by the Institutional Review Board (IRB) of Partner’s Healthcare System. PMN concentrations were adjusted to 5 × 10⁶ cells per ml in minimum essential medium supplemented with 1% bovine serum albumin (MEM 1% BSA). To remove endogenous IgG from the complement source (baby rabbit serum; Cedarlane Laboratories Ltd.), 1 ml was adsorbed 5 times at 4°C for 30 min with continual mixing with protein G-magnetic beads (Millipore, Bedford, MA) by following the manufacturer’s instructions. After adsorption, the complement solution was filter sterilized. NRS and anti-9Glc-NH₂-TT sera were diluted 1:10 in MEM 1% BSA and absorbed at 4°C for 30 min with an A. baumannii PNAG-negative strain, S1 Δpga, to remove
antibodies to this organism not directed to the PNAG antigen. The bacteria were removed by centrifugation, and both NRS and anti-9Glc-NH$_2$-TT serum were heated at 56°C for 30 min to inactivate endogenous complement activity and then were filter sterilized. The bacterial strains to be evaluated in the opsonophagocytic killing assay (OPKA) were grown in LB to logarithmic phase as determined by achieving an optical density at 650 nm (OD$_{650}$) of 0.4 (~2 × 10$^5$ CFU/ml) and a 1:100 dilution made in MEM 1% BSA for use in the OPKA.

The actual OPKA was performed by mixing 100 μl of PMNs, target bacteria, dilutions of the test antibodies, and the complement source. The reaction mixture was incubated on a rotor rack providing end-over-end rotation at 37°C for 90 min. Samples were taken at time zero and after 90 min of incubation, and they were plated for bacterial enumeration by making 10-fold dilutions in LB with 0.05% Tween to inhibit bacterial growth. The actual OPKA was performed by mixing 100 μl of PMNs, target bacteria, dilutions of the test antibodies, and the complement source. The reaction mixture was incubated on a rotor rack providing end-over-end rotation at 37°C for 90 min. Samples were taken at time zero and after 90 min of incubation, and they were plated for bacterial enumeration by making 10-fold dilutions in LB with 0.05% Tween to inhibit bacterial growth.

RESULTS
PNAG on A. baumannii is a target of opsonic antibodies. The OPKA is a well-established in vitro test that measures antibody function and can be a good surrogate assay for immune protection in vivo (18). We used the OPKA to investigate the ability of rabbit sera to 9Glc-NH$_2$-TT to promote the killing of our prototype high-PNAG-producing A. baumannii strain S1, the isogenic PNAG-negative mutant wherein the pga locus (10) was deleted (Δpga), and the Δpga mutant complemented in trans with an intact pga locus in the presence of rabbit complement and human PMNs. As presented in Fig. 1, rabbit sera to the 9Glc-NH$_2$-TT conjugate vaccine exhibited a high OPK activity against wild-type A. baumannii S1 (83.4% killing) but not against its PNAG-negative mutant strain S1 Δpga (3.2% killing). The complementation of the S1 Δpga strain with the pgaABCD genes in trans resulted in high levels of opsonic killing (76.1%) that were comparable to those seen with the wild-type S1 strain. As controls in the OPKA, we included tubes containing bacteria incubated with

![FIG 1](http://iai.asm.org)
of surface PNAG (10). The levels of PNAG among these clinical isolates rank as follows: S13, S26, S28, and S29, in the presence of human PMNs and rabbit complement. Control tubes containing bacteria, complement, and PMNs were included in every assay, and growth occurred in all instances. Bars represent the averages from three independent experiments, and error bars represent the SEM.

PMNs, complement, and NRS instead of 9Glc-NH$_2$-TT or tubes containing PMNs, antisera to 9Glc-NH$_2$-TT, and heat-inactivated complement. In these control tubes, bacterial growth rather than killing was always seen after 90 min of incubation. This was true for all three A. baumannii S1 isogenic strains (Fig. 1). These results highlight the specificity of the killing activity due to antibodies to PNAG as well as demonstrate a requirement for intact complement in the killing activity of antisera raised to 9Glc-NH$_2$-TT.

This specificity of the killing activity of 9Glc-NH$_2$-TT to anti-PNAG antibodies is in agreement with previous results obtained in our laboratory that showed that the inclusion of the nonacetylated oligomer 11GlcNH$_2$ in the OPKA strongly inhibited the killing of a PNAG-producing Staphylococcus aureus strain by 9Glc-NH$_2$-TT antisera (12). Moreover, similarly to the results with A. baumannii in this study, we previously demonstrated that antisera to 9Glc-NH$_2$-TT specifically kill two PNAG-producing Escherichia coli strains but not an E. coli H1 strain that did not synthesize PNAG (12). Taken together, these results further underscore the notion that antibodies to PNAG are responsible for the killing activity of anti-9Glc-NH$_2$-TT antisera.

**Opsonic activity of an antisem to 9Glc-NH$_2$-TT against various A. baumannii clinical isolates.** Since PNAG on strain S1 was an effective target for opsonic killing antibody, we decided to test the OPK activity of antisera to 9Glc-NH$_2$-TT against a panel of four MDR A. baumannii clinical isolates, S13, S26, S28, and S29, all of which were previously determined to express various levels of surface PNAG (10). The levels of PNAG among these A. baumannii clinical isolates rank as follows: S13 > S28 > S29 = S26 (10). As shown in Fig. 2, all four MDR isolates of A. baumannii tested in the OPKA, strains S13, S26, S28, and S29, were highly susceptible to killing by a rabbit antisem raised to 9Glc-NH$_2$-TT in the presence of rabbit complement and human PMN cells (82 to 94% at an antibody dilution of 1:10). Similarly high levels of opsonic killing were observed when the 9Glc-NH$_2$-TT antisem was tested at a 1:20 dilution (77 to 88% killing), with the exception of strain S29, which showed significantly lower levels of killing at this serum dilution. As an additional control, we also included in every assay tubes containing bacteria, complement, and PMNs lacking antibodies, and bacterial growth was always observed after 90 min of incubation.

With the exception of strain S29 at a 1:20 serum dilution, there were no significant differences in the killing of all four A. baumannii strains by antisera to PNAG at 1:10 or 1:20 in the OPKA, which indicated that OPK was independent of the level of PNAG production and that only a small amount of surface PNAG is necessary to mediate OPK activity of antibodies to 9Glc-NH$_2$-TT. In a similar manner, the Staphylococcus aureus strain MN8, which elaborates very little PNAG as detected by immunoblotting, has consistently been shown to be efficiently killed by antibodies to PNAG in the OPKA (23).

Taken together, our results demonstrate the ability of antisera raised to a synthetic oligosaccharide of PNAG conjugated to TT to opsonize and kill multiple unrelated MDR clinical strains of A. baumannii expressing a range of surface PNAG levels, supporting the potential of PNAG-specific antisera to confer protective immunity in vivo.

**Protective efficacy of an antisem to 9Glc-NH$_2$-TT in pneumonia and bacteremia models of A. baumannii infection.** We used two clinically relevant models of A. baumannii infection, pneumonia and bacteremia, to test the protective efficacy of antibodies to PNAG. Mice were passively immunized with either nonabsorbed antibody to PNAG or NRS as the control serum before infection. Nonabsorbed rabbit sera were used in animal protection studies to ascertain that natural antibodies in rabbit serum do not offer any protective activity in vivo and to avoid introducing bacterial LPS and other innate immune-activating molecules into the absorbed sera that could lead to an increase in nonspecific resistance to infection.

For the pneumonia and bacteremia protection studies, mice were challenged i.n. and i.v., respectively, with our prototype high-PNAG-producing strain S1 and three additional MDR clinical isolates of A. baumannii, S13, S28, and S29, which had been previously tested in the OPKA. A. baumannii S1 Δpga also was used as a control. Results presented in Fig. 3 and 4 represent the bacterial burden in the lungs or blood of mice 24 or 2 h postinfection, respectively, in animals given antibody to PNAG or control NRS. As it became apparent from these studies, depending on the A. baumannii strain tested, we found either an increase in bacterial CFU in tissues or some clearance of the inocula postinfection in immunimune mice. These variations reflect the expected differences in virulence among A. baumannii clinical isolates in our, or any, pneumonia and bacteremia models of infection (Fig. 3 and 4). It also was interesting that the bacterial burdens in the lungs and blood of wild-type A. baumannii S1-infected and PNAG-negative mice were comparable, indicating that PNAG is not virulence factor in pneumonia and bloodstream infection models.

Mice immunized with the antisem raised to 9Glc-NH$_2$-TT and subsequently infected with four A. baumannii clinical isolates had significantly lower numbers of CFU per g of lung tissue 24 h postinfection than animals receiving nonimmune rabbit serum (Fig. 3). This was true for all four A. baumannii strains tested (S1, P < 0.0001; S26, P = 0.0016 by unpaired t test; S13, P = 0.012; and S29, P = 0.0006 by t test with Bonferroni correction). On the other hand, when we used the same pneumonia model but challenged animals with the S1 Δpga strain at a dose similar to the one used with the wild-type S1 strain, no significant differences were observed in CFU/g of lung tissue between animals given the antisem.
FIG 3 Passive immunization of mice with rabbit antisera raised to 9Glc-NH₂-TT led to significantly reduced levels of *A. baumannii* strains in lung tissues compared to levels of mice subjected to NRS. Mice (n = 7 to 26 per group) were infected i.n. with *A. baumannii* S1 (2.3 × 10⁵ CFU/mouse), S13 (3.7 × 10⁴ CFU/mouse), S26 (1.5 × 10⁵ CFU/mouse), or S29 (5.9 × 10⁵ CFU/mouse). *A. baumannii* S1 Δpga (1.2 × 10⁵ CFU/mouse) was used as a specificity control. Bars indicate the mean CFU per g of lung tissue, and error bars indicate the SEM. t tests were used for comparisons between control and immune groups for all strains except for strain S1 in the protection studies, where two replicate experiments were carried out and comparisons made by t test with the Bonferroni correction. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant (P > 0.05).

FIG 4 Passive immunization with rabbit antisera raised to 9Glc-NH₂-TT significantly reduced levels of *A. baumannii* surviving in the blood 2 h after i.v. injection compared to levels for mice subjected to NRS. Mice (n = 7 to 15) were injected i.v. with *A. baumannii* strain S1 (2.3 × 10⁵ CFU/mouse), S13 (3.7 × 10⁴ CFU/mouse), S26 (1.5 × 10⁵ CFU/mouse), or S29 (5.9 × 10⁵ CFU/mouse). *A. baumannii* S1 Δpga (1.2 × 10⁵ CFU/mouse) was used as a specificity control. Bars indicate the mean CFU per ml of blood, and error bars indicate the SEM. t tests were used for comparisons between control and immune groups for all strains except for strain S1 in the protection studies, where two replicate experiments were carried out and comparisons made by t test with the Bonferroni correction. *, P < 0.05; ns, not significant (P > 0.05).

The optimal time point to measure bacterial clearance. Using the same four *A. baumannii* clinical isolates for the bacteremia studies that were used in the pneumonia studies, mice immunized i.v. 24 and 4 h prior to challenge with the antisera to 9Glc-NH₂-TT had significantly lower bacterial levels in the bloodstream 2 h after infection than animals given NRS (S1, P = 0.018 by t test with Bonferroni correction; S13, P = 0.0153; S26, P = 0.0382; and S29, P = 0.013 by unpaired t test). Conversely, when mice were infected i.v. with *A. baumannii* strain S1 Δpga at a dose similar to the one used for wild-type *A. baumannii* S1, we found no significant differences in the CFU/ml of blood between immunized and control groups (P = 0.69 by unpaired t test) (Fig. 4).

Taken together, these results demonstrate the protective efficacy of antisera to 9Glc-NH₂-TT in murine pneumonia and bacteremia models of *A. baumannii* infection.

**DISCUSSION**

Although *A. baumannii* generally is regarded as a low-virulence pathogen (11, 29), it causes infections in well-defined high-risk populations, such as patients receiving mechanical lung ventila-
tion, residents of long-term care facilities, and wounded military personal (16, 26, 33). These infections are complicated by the recent emergence of MDR or even pandrug-resistant (PDR) isolates that are very difficult to eradicate. While the targeted vaccination of these specific, high-risk populations via active immunization is feasible, this group of patients is difficult to identify prospectively. Thus, based on the urgent need for better therapies for A. baumannii infections, we evaluated the efficacy of passive immunization against the conserved surface polysaccharide PNAG as a means to prevent A. baumannii infections in patients at high risk for acquiring this pathogen. Overall, we found antibody to PNAG, raised using a synthetic oligosaccharide conjugate vaccine, mediated high levels of the opsonic killing of multiple A. baumannii strains and significantly reduced levels of bacteria in the lungs and blood of mice following infection.

We observed a correlation between the opsonic killing of anti-PNAG antibodies and their in vivo protective efficacy in the pneumonia model of infection and, to a lower extent, in the bacteremia infection model. The levels of bacterial clearance by anti-9Glc-NH₂-TT from the lungs of mice infected with A. baumannii S1, S13, S26, and S28 were 73, 73, 81, and 59.7%, respectively, and they correlated well with the levels of killing of the same four strains in OPKA that ranged from 82 to 98% at a 1:10 serum dilution and 77 to 88% at 1:20 for the strains S13, S26, and S28, since S1 was never tested in the OPKA at a 1:20 serum dilution.

For the bacteremia protection studies, the levels of clearance by antisera to 9Glc-NH₂-TT (45.5 to 66.3%) were somewhat lower than those seen with the pneumonia studies but still were significantly different from those for the NRS control.

In our extensive experience with various murine models of A. baumannii infection, including pneumonia, bacteremia, and wound infections, we have consistently observed that A. baumannii is a low-virulence pathogen in these animal models, even in the setting of neutropenia. One major limitation of the A. baumannii infection models is that even in the nonimmune mice, which are quite healthy, there is little in vivo replication, and animals clear a large proportion of the inoculum. Therefore, it becomes more challenging to observed significant differences in response to immunization, but showing an in vivo effect of our antibodies represents a critical preclinical evaluation of potential efficacy in human infections. Enhanced reductions in bacterial loads is an indicator of potential efficacy, showing that the cofactors needed by the antibody, phagocytes and complement, are present at levels in the murine lung sufficient to lead to enhanced reduction in the bacterial load in the presence of antibody to PNAG. These findings establish a proof of principle for targeting PNAG expressed by A. baumannii using immunotherapy approaches.

Although PNAG did not appear to be a virulence factor in the setting of acute pneumonia or bacteremia in mice, several properties of PNAG related to virulence functions support the utility of developing immunotherapies targeting this antigen on A. baumannii (10). The elaboration of PNAG is critical for biofilm formation by this pathogen (10). Moreover, the pga genes encoding the PNAG biosynthetic enzymes were present in all 30 clinical isolates examined previously (10), and 28 of these 30 expressed detectable PNAG on their surface (10), indicating that PNAG production is widely conserved among A. baumannii clinical isolates. This conservation of PNAG expression among A. baumannii clinical isolates enhances its value as a vaccination target.

While new therapies and approaches for preventing or treating MDR and PDR pathogens such as A. baumannii are a high priority, it would be challenging to develop vaccines or immunotherapies specific for this pathogen or to evaluate them in a proper clinical trial, because A. baumannii infections tend to be sporadic within different hospitals. However, in certain health care institutions A. baumannii infections are endemic and very difficult to eradicate, and they are likely common enough for a proper trial of antibody-based approaches.

Furthermore, PNAG is expressed by a broad range of bacterial pathogens, including S. aureus, Staphylococcus epidermidis (23–25), and Escherichia coli (14, 34), all of which are major nosocomial pathogens. Thus, evaluations of immunotherapies to PNAG for these pathogens might make it possible to include an evaluation of the occurrence of A. baumannii infections in treated patients. Alternatively, demonstrating the clinical efficacy of immunotherapy against PNAG for the more common pathogens expressing this antigen could support the utility, in the absence of a full clinical trial, of using these antibodies against less common organisms like A. baumannii, particularly in situations where isolates are highly resistant to antibiotics.

While prior results with S. aureus and E. coli establish that antibodies to the deacetylated version of PNAG, termed dPNAG, and its oligosaccharide analog, 9Glc-NH₂, mediate in vitro opsonic killing and protect mice from infections (9, 23), it cannot be readily assumed that these antibodies will be universally efficacious against all PNAG-producing pathogens. The fine structure of PNAG varies among bacterial species and even isolates of the same species (10, 22, 34), making it critical to test each PNAG-producing pathogen, which express their own unique set of virulence factors and capabilities to cause infections, against antibodies to the dPNAG or 9Glc-NH₂ glycoforms for susceptibility to killing and protective efficacy. In this study, we validated that the 9Glc-NH₂ oligosaccharide conjugated to TT induced opsonic and protective antibodies to four MDR strains of A. baumannii, providing support for further investigations of active and passive immunizations using deacetylated glycoforms of PNAG as antigens for protection against the range of pathogens producing this conserved surface polysaccharide.

ACKNOWLEDGMENTS

We acknowledge Tse Hsien Koh for providing a collection of MDR clinical isolates of A. baumannii.

G.B.P. and T.M.-L. declare financial interests in the development of PNAG-based vaccines and immunotherapies. They are inventors of technology for both active vaccination with PNAG-based vaccines and passive immunotherapy with monoclonal antibodies directed to the PNAG antigen. The antibody has been licensed by Brigham and Women’s Hospital to Alopexx Pharmaceuticals, a company that G.B.P. cofounded and owns. As inventors, G.B.P. and T.M.-L. receive a share of licensing-related income (royalties and fees) through Brigham and Women’s Hospital. These financial interests were reviewed and are managed by Brigham and Women’s Hospital and Partners HealthCare in accordance with their conflict of interest policies.

This work was supported by grants from the National Institutes of Health, National Institute of Allergy and Infectious Diseases, AI46706 (G.B.P.) and AI057159 (G.B.P.), a component of award U54 AI057159.

The content is solely the responsibility 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.
REFERENCES


