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Research Paper

The pneumococcal alpha-glycerophosphate oxidase enhances nasopharyngeal colonization through binding to host glycoconjugates

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ABSTRACT

Streptococcus pneumoniae (the pneumococcus) is a major human pathogen, causing a broad spectrum of diseases including otitis media, pneumonia, bacteraemia and meningitis. Here we examined the role of a potential pneumococcal meningitis vaccine antigen, alpha-glycerophosphate oxidase (*Sp*GlpO), in nasopharyngeal colonization. We found that serotype 4 and serotype 6A strains deficient in *Sp*GlpO have significantly reduced capacity to colonize the nasopharynx of mice, and were significantly defective in adherence to human nasopharyngeal carcinoma cells *in vitro*. We also demonstrate that intranasal immunization with recombinant *Sp*GlpO significantly protects mice against subsequent nasal colonization by wild type serotype 4 and serotype 6A strains. Furthermore, we show that *Sp*GlpO binds strongly to lacto/neolacto/ganglio host glycan structures containing the GlcNAcβ1-3Galβ disaccharide, suggesting that *Sp*GlpO enhances colonization of the nasopharynx through its binding to host glycoconjugates. We propose that *Sp*GlpO is a promising vaccine candidate against pneumococcal carriage, and warrants inclusion in a multi-component protein vaccine formulation that can provide robust, serotype-independent protection against all forms of pneumococcal disease.

1. Introduction

Streptococcus pneumoniae (the pneumococcus) continues to be responsible for major morbidity and mortality worldwide, causing a broad spectrum of diseases including otitis media (OM), pneumonia, bacteraemia and meningitis (WHO, 2014a, 2015). The prevalence of antibiotic-resistant pneumococci is increasing rapidly (WHO, 2014b), and available capsular-based vaccines are expensive and have major shortcomings with respect to immunogenicity, serotype replacement and/or strain coverage (Olarte et al., 2015). This has stimulated global efforts towards the development of cheaper, non-serotype-dependent vaccines based on conserved pneumococcal virulence proteins. However, protein antigens currently under consideration are being selected solely on the basis of their capacity to elicit protection in models of pneumonia and bacteremia, but their ability to protect against colonization of the nasopharynx, OM and meningitis is unproven (Ogunniyi and Paton, 2015).

Asymptomatic colonization of the nasopharynx almost invariably precedes disease; however, pneumococci vary in their capacity to colonize the nasopharynx, both in humans and in animal models. Certain serotypes and/or clonal groups are more often isolated from carriers, while others are more often isolated from sterile sites (Brueggemann et al., 2003; Sandgren et al., 2004; Sjostrom et al., 2006). In the context of rational pneumococcal protein vaccine design, an ideal vaccine formulation will reduce colonization as well as prevent invasive pneumococcal disease (Paton and Ogunniyi, 2011). A number of pneumococcal proteins have been shown to protect against colonization in addition to their protective capacities against invasive disease in animal models. Foremost among these are maltose/maltodextrin ABC transporter binding protein (MalX) (Moffitt et al., 2011), Neuraminidase A (NanA) (Tong et al., 2005), and pneumococcal surface proteins A and C (PspA and PspC) (Balachandran et al., 2002; Wu et al., 1997). Nevertheless, novel candidate proteins continue to be identified and appraised for inclusion in multi-component pneumococcal protein vaccines that are currently under development.

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In a series of investigations, we used *in vivo* gene expression and microarray analyses to identify the factors that trigger progression from colonization to invasive or meningeal infection in a murine model. One of these genes, *glpO*, encoding the pneumococcal alpha-glycerophosphate oxidase (herein designated *Sp*GlpO), was found to be upregulated in the brain *versus* blood of mice, and contributes significantly to the development of meningitis. Preliminary analysis also revealed that a pneumococcal *glpO* deletion mutant was significantly attenuated for nasopharyngeal colonization relative to the isogenic wild type (Mahdi et al., 2012). Given this important finding, we carried out a detailed analysis of the role of *Sp*GlpO in colonization, assessed its vaccine potential against pneumococcal carriage in a murine model, and examined its role in the host-pathogen interaction.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

The pneumococcal strains used in this study were serotype 6A (WCH16), serotype 4 (WCH43), and their isogenic Δ*glpO* mutant derivatives (Table S1). Serotype-specific capsule production was confirmed by Quellung reaction, as described previously (Berry and Paton, 2000). Bacteria were grown statically at 37 °C in serum broth (SB; 10% heat-inactivated horse serum in nutrient broth) to $A_{600\ nm}$ of 0.16 (equivalent to approx. 5×10^7 CFU/ml).

2.2. Mice

Outbred 5- to 6-week-old female CD1 (Swiss) mice, obtained from the Laboratory Animal Services specific pathogen-free breeding facility of The University of Adelaide, were used in all experiments and were housed in the same facility for the entire duration of the experiments. Animals were provided with food and water *ad libitum*. The Animal Ethics Committee of The University of Adelaide approved all animal experiments (approval numbers S-2010-001 and S-2013-053). The study was conducted in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition 2004 and 8th Edition 2013) and the South Australian Animal Welfare Act 1985. Animal experimentation were conducted in accordance with ARRIVE guidelines for animal research.

2.3. Mixed Infection Experiments

For *in vivo* competition experiments, mutant and wild type bacteria were mixed at an input ratio of 1:1, and groups of mice were challenged intranasally (i.n.) under anesthesia with 50 μl mixed bacterial suspension containing approx. 2.5×10^6 CFU each strain in SB. At 48 and 72 h post-challenge, 14–16 mice from each mixed inoculum experiment were sacrificed, and the bacterial load for each strain in the nasopharynx was quantitated as described previously (Ogunniyi et al., 2012). A 40 μl aliquot of each sample was serially diluted in SB and plated on blood agar and blood agar with a selective antibiotic marker (spectinomycin resistance) carried by the Δ*glpO* deletion mutant to determine the ratio of mutant to wild type bacteria. Competitive indices were calculated as the ratio of mutant to wild type bacteria recovered, adjusted by the input ratio. Differences in colonization between wild type and mutant were analyzed by one-sample *t*-test (two-tailed). A P value of <0.05 was considered statistically significant.

2.4. Adherence Assays

Adherence of pneumococci to human nasopharyngeal carcinoma epithelial (Detroit 562) cells (ATCC® CCL 138™) was assayed essentially as described previously (Mahdi et al., 2012). Cells were maintained in a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM, and Ham's F-12 medium (Gibco; Cat No: 11320–033), supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 25 mM HEPES, 100 U per ml penicillin-streptomycin. Strains were grown in C + Y medium (Lacks and Hotchkiss, 1960) supplemented with 1% glycerol (instead of glucose) for maximal *Sp*GlpO expression and diluted to approximately 2×10^6 CFU/ml in serum-free medium (without antibiotics). 1 ml aliquots of bacteria were inoculated in triplicate onto washed confluent monolayers (approx. 2×10^5 cells) in 24-well tissue culture trays and incubated at 37 °C in 5% CO_2 for 2 h. In addition, aliquots of wild type and mutant bacteria were treated before inoculation with 2.5 μl of high titer (1:50,000) polyclonal mouse anti-*Sp*GlpO serum. Following adherence, cell monolayers were washed 3 times with PBS and released and resuspended using 100 μl of 0.25% Trypsin-EDTA (Gibco; Cat No: 25200056) for 2 min after which 400 μl of 0.025% Triton X 100 (Sigma; Cat No: T8787) was added for 5 min to lyse Detroit 562 cells and release bacteria. Samples were resuspended by repeated pipetting and adherent bacteria were quantiated by serial dilution and plating on BA overnight at 37 °C in 5% CO_2. Assays were performed in triplicate in two independent experiments. Differences between means were analyzed using the unpaired Student's *t*-test (two-tailed).

2.5. Glycan Array

Glycan arrays were produced from a library consisting of 367 diverse glycans with and without one of three spacers (sp2, sp3 or sp4) (Blixt et al., 2004) made up of two previously described glycan libraries (Arndt et al., 2011; Huflejt et al., 2009). Glycans were either amine functionalized with spacers sp2, sp3 or sp4 as previously described (Blixt et al., 2004) or without spacers as previously published (Day et al., 2009). Glycan arrays were printed as previously published (Waespy et al., 2015). Glycan array experiments were performed and analyzed as previously described (Shewell et al., 2014) using 2 μg of purified *Sp*GlpO per array.

2.6. Surface Plasmon Resonance (SPR) Analyses

SPR analyses were performed using a Biacore T100 System (GE Healthcare Life Sciences) at 25 °C at a flow rate of 30 μl per min. Purified *Sp*GlpO was diluted to 50 μg per ml in PBS and loaded on the appropriate flow cells of a Ni^{2+} NTA sensor chip with 5 min contact time. Flow cell 1 in each run contained an unrelated His-tagged protein, which was tested to ensure it did not bind glycans, and was used as a reference. Glycans analyzed for interactions with *Sp*GlpO were serially diluted from 200 μM to 0.32 μM in PBS and analyzed as previously described (Shewell et al., 2014).

2.7. Inhibition of Adherence by Sugars

Inhibition of adherence of wild type pneumococci and their isogenic Δ*glpO* mutant derivatives to Detroit 562 cells by asialo-GM1 (aGM1; Cat No: GLY102, Elicityl OligoTech) trisaccharide, lacto-*N*-neotetraose tetrasaccharide (LNnT; Cat No: GLY021, Elicityl OligoTech) and lacto-*N*-tetraose tetrasaccharide (LNT; Cat No: GLY010, Elicityl OligoTech) was carried out as follows. Each bacterial strain (2×10^5 CFU) was pre-incubated with 560 μM of each sugar (in duplicate) for 2 h at 37 °C in 5% CO_2 before addition to duplicate Detroit 562 cell monolayers in 24 well plates containing 2×10^4 cells in antibiotic-free 1:1 DMEM and Ham's F-12 medium + 10% FCS). Trays were incubated for a further 2 h at 37 °C in 5% CO_2 after which monolayers were washed 3 times in PBS and treated with 25 μl of 0.25% Trypsin for 2 min and then 100 μl of 0.025% Triton X-100 for 5 min. Samples were then plated on BA and incubated overnight at 37 °C in 5% CO_2 for bacterial enumeration. Assays were performed in duplicate in two independent experiments. Differences between means were analyzed using the unpaired Student's *t*-test (two-tailed).

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