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Contributions of capsule, lipoproteins and duration of colonisation towards the protective immunity of prior *Streptococcus pneumoniae* nasopharyngeal colonisation

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ABSTRACT

Live attenuated vaccines have been proposed as a strategy to induce protective immunity against infectious diseases. Recent data have demonstrated that nasopharyngeal colonisation with *Streptococcus pneumoniae* induces protective immunity against subsequent invasive infection, suggesting nasal vaccination with live attenuated bacteria could be a preventative strategy. However the bacterial factors affecting the strength of this adaptive immune response remain unclear. In a direct comparison with the parent wild-type strain, we found that colonisation with bacteria lacking either capsule or surface lipoproteins led to significantly diminished protection. Immunity after colonisation was not dependent on serum IgG to capsular antigens. Colonisation density and duration was reduced for all the non-protective strains, suggesting that protective immunity maybe related to the extent of nasopharyngeal bacterial exposure. To investigate this hypothesis, we utilised an auxotrophic bacterial Δ*pab* strain where duration of colonisation could be controlled by supply and removal of para-amino-benzoic acid (PABA) to mouse drinking water. Supporting colonisation with the Δ*pab* strain for 5 days with PABA led to a faster serum antibody response compared to colonisation for less than 48 h. This enhanced immunogenicity was associated with a trend towards protection. The data presented here aid our understanding of why only certain live attenuated strains are able to function as effective vaccines, and may be valuable in informing the constituents of future live attenuated vaccines.

1. Introduction

Streptococcus pneumoniae is a global pathogen responsible for the deaths of over one million individuals annually, mostly due to pneumonia [1]. Initial exposure to the bacteria is in the nasopharynx, where they establish colonisation. Usually, episodes of nasopharyngeal colonisation are essentially asymptomatic, and do not lead to disease [2]. In certain cases however, when the range of innate and adaptive immune mechanisms is insufficient to prevent disease, aspiration of bacteria can lead to pneumonia. This is most common at the extremes of life and amongst immunocompromised individuals. Vaccines have been directed to this specific need.

At present, licensed vaccines elicit protection through induction of opsonophagocytic antibodies against capsular polysaccharide antigens [3]. Once conjugated to carrier proteins, a process necessary to induce protection in infants, these vaccines can lead to reduction in carriage as well as disease. These conjugate vaccines are very effective at reducing disease caused by the *S. pneumoniae* serotypes included in the vaccine directly in the vaccinees and indirectly in the wider community. However, serotypes not included in the vaccine can replace the eliminated strains within the nasopharynx, leading to replacement disease [4]. Despite recent increases in the number of serotypes included in vaccine formulations, it is likely that alternative strategies will be required in the long-term to protect against *S. pneumoniae* [3].

Live vaccines can lead to both humoral and cellular immune responses. Inclusion of a large number of antigens and natural bacterial adjuvants can lead to strong immunity in the absence of an

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exogenous adjuvant. Nasopharyngeal colonisation with live bacterial strains represents one such route of mucosal immunisation.

Using murine models, we [5] and others [6,7] have studied the mechanisms by which prior colonisation can protect against subsequent lethal invasive pneumonia. Antibody responses induced through colonisation with a live wild-type (WT) strain are both necessary and sufficient to protect against invasive disease [5]. Such protection does not necessarily require antibodies to capsular polysaccharide, since experimental colonisation with unencapsulated strains is also protective [6]. Unencapsulated mutants are an attractive option for live attenuated vaccines due to their lack of virulence [6,8], but no direct comparison of the immunogenicity and protective efficacy of colonisation with isogenic strains with and without capsule has been reported.

Bacterial lipoproteins are an important class of pathogen-associated molecular pattern (PAMP), capable of adjuvanting immune responses [9] by acting as ligands for TLR2 [10], and are common targets for adaptive immune responses [11,12]. Deletion of *lgt*, which encodes the protein diacylglyceryl transferase required to anchor lipoproteins to the cell membrane, results in an *S. pneumoniae* strain with greatly reduced quantities of lipoproteins on the bacterial surface (Chimalapati, under review) [13]. This Δ*lgt* strain is still able to colonise the mouse nasopharynx, albeit with both reduced density and shorter duration than its parent WT strain. Its ability to induce protective immunity is not known.

The gene *pabB* encodes para-amino benzoic acid (PABA) synthase, required for the folate biosynthetic pathway. Deletion of this gene leads to an auxotrophic mutant where growth is dependent upon exogenous supply of PABA [11]. It is unlikely to affect capsule expression since phagocytosis of the Δ*pab* strain *in vitro* is similar to that of its parent strain [11]. The Δ*pab* mutation does not significantly effect lipoprotein expression, since such strains can robustly induce anti-lipoprotein antibodies when inoculated *via* the intraperitoneal route [11]. This mutation results in an inability to replicate *in vivo*, and was previously reported to lead to rapid clearance of TIGR4Δ*pab* from the nasopharynx within 2 days. This mutant was also avirulent unless the animal's drinking water was supplemented with PABA [11]. Again, its ability to induce protection through colonisation is not known.

In this study, we address the specific contribution of the presence of capsule and surface lipoproteins on colonisation-induced immunogenicity and protection against subsequent lethal pneumonia. We find that absence of either capsule or lipoproteins leads to failure to protect, reflecting reduced immunogenicity. Using controlled colonisation with an auxotrophic mutant, we find that duration and density of colonisation directly impacts on the speed of the immune response, with potential impact on subsequent protection.

2. Methods

2.1. Ethics statement

Experiments were approved by the UCL Biological Services Ethical Committee and the UK Home Office (Project Licence PPL70/6510). Experiments were performed according to UK national guidelines for animal use and care, under UK Home Office licence and in accordance with EU Directive 2010/63/EU.

2.2. Bacterial strains and culture conditions

Wild-type (WT) *S. pneumoniae* strain D39 (serotype 2) and its unencapsulated derivative containing a deletion of *cpsD* (D39-DΔ) [14] were a kind gift from James Paton, University of Adelaide. Deletional mutant strain D39Δ*pab* lacking PAB synthetase or *lgt* were generated by overlap extension PCR as described [11] (Chimalapati, under review). Bacteria were cultured on Columbia agar with 5% horse blood or in Todd–Hewitt broth with 0.5% yeast extract in 5% CO_2. Inocula for challenge experiments were prepared from mid-log phase cultures and stored at −70 °C as single use aliquots.

2.3. Colonisation and infection models

CD1 outbred mice were obtained from Charles River UK Ltd. Mice were colonised by instillation of 10^7 cfu *S. pneumonia* in 10 μl PBS into the nares under light halothane anaesthesia as previously [5,15]. In certain experiments, mice received a second colonising dose 2 weeks after the first dose. Control mice received 10 μl PBS alone. To obtain nasal washes the exposed trachea was flushed caudally with 200 μl PBS and the fluid exiting the nares collected. For the pneumonia challenge, 10^7 cfu *S. pneumonia* D39 in 50 μl PBS was instilled into the nares under deep general halothane anaesthesia 28 days after the final colonising dose [5,15,16]. Animals were culled by exsanguination from the femoral artery under pentobarbital anaesthesia. Broncheo-alveolar lavage fluid (BALF) was collected by cannulating the exposed trachea and washing the airways three times serially with 1 ml sterile PBS. Lungs were collected aseptically into ice-cold PBS, minced and homogenised with sterile PBS as previously [5,17]. For survival experiments, animals were monitored and culled when exhibiting previously defined features of terminal disease [16].

2.4. Whole cell ELISA

Antibodies specific to antigens in different *S. pneumoniae* strains were measured by whole cell ELISA using established methods as previously described [8]. Briefly, *S. pneumoniae* were grown to late log-phase, washed and resuspended in PBS to OD_{580} 1.0. 96-well plates were coated with this bacterial suspension, refrigerated overnight, then blocked with PBS 1% BSA prior to use. Sera were diluted in PBS 1% BSA before addition and binding to bacterial antigens detected with anti-mouse secondary antibodies conjugated to alkaline phosphatase (Sigma).

2.5. Capsule ELISA

To measure capsule-specific antibodies, plates were coated with type 2 purified capsular polysaccharide (CPS) at 10 μg/ml (LGC Promochem). To increase assay specificity, sera were pre-incubated with cell wall polysaccharide (Statens Serum Institut) and type 22F capsular polysaccharide (LGC Promochem) as previously [11]. Development of ELISAs proceeded as for whole cell ELISAs.

2.6. Competitive inhibition ELISA

To determine the relative contribution of CPS binding towards the total binding observed in whole cell ELISA, sera were pre-incubated in PBS/1% BSA with increasing concentrations of soluble type 2 CPS up to 100 μg/ml for 30 min at RT, prior to assay in whole cell ELISA as above.

2.7. Luminex anti-pneumococcal protein antibody assay

Antibody responses to multiple protein antigens were measured using a multiplex flow cytometry Luminex assay based on *S. pneumoniae* proteins conjugated to xMAP beads, as previously [11]. Recombinant TIGR4-, D39-, or serotype 23 strain-derived proteins were conjugated to xMAP beads (Luminex) [18]. Combined beads (3000 per antigen) were incubated with 10% or 1% serum in PBS–1% bovine serum albumin and then with goat anti-mouse IgG-phycoerythrin (Jackson ImmunoResearch). IgG binding was