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Genetic conjugation of components in two pneumococcal fusion protein vaccines enhances paediatric mucosal immune responses

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ABSTRACT

Streptococcus pneumoniae colonises the upper respiratory tract and can cause pneumonia, meningitis and otitis media. Existing pneumococcal conjugate vaccines are expensive to produce and only protect against 13 of the 90+ pneumococcal serotypes; hence there is an urgent need for the development of new vaccines. We have shown previously in mice that pneumolysin (Ply) and a non-toxic variant (Δ6Ply) enhance antibody responses when genetically fused to pneumococcal surface adhesin A (PsaA), a potentially valuable effect for future vaccines. We investigated this adjuvanticity in human paediatric mucosal primary immune cell cultures. Adenoidal mononuclear cells (AMNC) from children aged 0–15 years ($n = 46$) were stimulated with conjugated, admixed or individual proteins, cell viability and CD4+ T-cell proliferative responses were assessed using flow cytometry and cytokine secretion was measured using multiplex technology.

Proliferation of CD4+ T-cells in response to PsaAPly, was significantly higher than responses to individual or admixed proteins ($p = 0.002$). In contrast, an enhanced response to PsaAΔ6Ply compared to individual or admixed proteins only occurred at higher concentrations ($p < 0.01$). Evaluation of cytotoxicity suggested that responses occurred when Ply-induced cytolysis was inhibited, either by fusion or mutation, but importantly an additional toxicity independent immune enhancing effect was also apparent as a result of fusion. Responses were MHC class II dependent and had a Th1/Th17 profile. Genetic fusion of Δ6Ply to PsaA significantly modulates and enhances pro-inflammatory CD4+ T-cell responses without the cytolytic effects of some other pneumolysoids. Membrane binding activity of such proteins may confer valuable adjuvant properties as fusion may assist Δ6Ply to deliver PsaA to the APC surface effectively, contributing to the initiation of anti-pneumococcal CD4+ T-cell immunity.

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1. Introduction

Streptococcus pneumoniae (pneumococcus), a frequent coloniser of the upper respiratory tract of young children, can cause invasive diseases such as meningitis and septicaemia as well as acute otitis media and pneumonia [1,2]. Current vaccines known to prevent pneumococcal infections in children consist of capsular polysaccharides conjugated to non-toxic carrier proteins [3,4]. However, the global impact of these vaccines is limited by cost, limited serotype coverage, ecological shifts as non-vaccine serotypes replace those diminished by vaccine use and relatively short duration of serum antibody responses, especially in young children [5–7]. Thus, intensive efforts are in progress to identify and develop alternative vaccines containing one or more pneumococcal

Abbreviations: TMNC, tonsillar mononuclear cell; AMNC, adenoidal mononuclear cell.

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protein antigens, which may offer broader, more sustained and more affordable protection.

As acquisition of pneumococcus, a pre-requisite for disease, is understood to occur via the upper respiratory tract, induction of protective nasopharyngeal immunity to either reduce acquisition or the density and/or duration of colonisation is an important goal for vaccine development. Bacterial toxins are promising candidates for inclusion in mucosal vaccines as they can display adjuvant properties, inducing strong immune responses to co-administered antigens [8,9]. Produced by virtually all clinical pneumococcal isolates [10] and a major virulence factor of *S. pneumoniae*, pneumolysin (Ply) is a 53 kDa cholesterol-dependent cytolysin (CDC) which binds to host-cell membranes and forms pores leading to cell lysis [11]. Ply is immunogenic [12] and natural immunity to it is acquired in children [13,14]. Studies in mice show that Ply recognition by toll-like receptor (TLR)-4 results in clearance of pneumococci from the nasopharynx and prevention of invasive disease [15,16]. Ply also promotes the production of IFN-γ, IL-17A and IL-1β, maturation of dendritic cells (DC's) and the activation of the NLRP3 inflammasome, all key processes in protection against pneumococcal infection [17].

Despite these potentially useful immunomodulatory properties, the cytolytic properties of Ply make its use in human vaccines complex. Pneumolysoids have been generated to reduce toxicity whilst preserving adjuvanticity and immunogenicity [18–20]. Δ6PLY has a double amino acid deletion (A146R147) in the region involved in oligomerisation and, unlike other Ply mutants, demonstrates good immunogenicity with no evidence of pore-formation [20] and does not induce proinflammatory activity associated with tissue damage in human neutrophils in vitro [21].

Pneumococcal surface adhesin A (PsaA), a highly conserved 35 kDa surface protein [22] is also a potential vaccine candidate [23,24] which induces mucosal immune responses in children [25]. By genetic conjugation a PsaAPly fusion protein was generated which, following intranasal immunisation in mice, induced significantly greater PsaA-specific immune responses than immunisation with PsaA alone, suggesting that, as part of a compound antigen, Ply can have mucosal adjuvant activity [26]. Also, antigen-specific responses were enhanced following immunisation with the non-cytolytic fusion protein, PsaAΔ6PLY [26]. Recent studies using alternative pneumolysoid fusion proteins have demonstrated their mucosal adjuvant potential [27] and ability to induce protection against pneumococcal colonisation and infection following immunisation in mice [27–29].

Here, using PsaAPly and PsaAΔ6PLY, we investigated whether the adjuvant effects of Ply observed in mice are also evident in adenoidal mononuclear cells from children.

2. Materials and methods

2.1. Subjects and tissue samples

Palatine tonsils and/or adenoids were obtained from 46 children aged 0–15 years undergoing routine adenoidectomy/adenotonsillectomy at the Bristol Royal Hospital for Children. Children were excluded from the study if they had any known immunodeficiency or had received treatment known to significantly suppress the immune system for ≥2 weeks prior to surgery. Ethical approval was obtained for the study from the local Research Ethics Committee (South West – Central Bristol) and from PATH. Written informed consent was obtained from all parents/guardians before enrolment.

2.2. Generation of proteins and fusion constructs

PsaAPly and PsaAΔ6Ply fusion protein constructs, PsaA, Ply and Δ6Ply were generated using In-fusion technology cloning (Clontech, France), as previously described [26].

2.3. Cell isolation and CFSE labelling

Adenoidal and tonsillar mononuclear cells (AMNC, TMNC) were isolated from resected tissue and cell viability was assessed as described previously [25]. To measure proliferation of cells AMNC and TMNC were labelled using carboxyfluorescein diacetate succinimidyl ester (CFSE) CellTrace proliferation kit (Life Technologies, UK) as described previously [14].

2.4. Cell culture and protein antigens

CFSE-labelled cells were re-suspended in complete RPMI (RPMI with 20 mM HEPES, 2 mM glutamine, 1% penicillin/streptomycin and 2% human serum (Sigma–Aldrich Company Ltd., UK)) and seeded at 1×10^6/ml in 48-well plates. Fused, admixed, or individual proteins were added to cells at equimolar concentrations as described in Table 1. Plates were incubated for 7 days at 37 °C, 5% CO_2.

HLA-DR blocking. For HLA-DR blocking, 5 μg/ml of purified mouse anti-human HLA-DR or mouse IgG2a isotype control antibodies (BD Biosciences, UK) were added to mononuclear cells on days 0 and 4 of culture.

2.5. Flow cytometry

On day 7, cells were labelled with an APC-conjugated mouse anti-human CD4 antibody (BD Biosciences, UK) and washed and stained with 7-Aminoactinomycin D (7-AAD) (Molecular Probes, USA) to allow the exclusion of dead cells from the analysis. For cell viability assays, cells were stained with 7-AAD only. Samples were then acquired using a FACSCantoII flow cytometer and data were analysed using FACSDiva software (BD Biosciences, UK).

2.6. Cytokine measurements

On day 7, supernatants were taken from cultures and stored at −80 °C. IL-17A, IFN-γ, IL-2, TNF-α, IL-10, IL-4 and IL-5 secretion were measured with a 7-plex human cytokine MILLIPLEX MAP kit according to the supplier's instructions (Millipore, UK). Plates were analysed using a Luminex 200™ instrument and xPONENT software (Luminex, USA).

2.7. Statistical analysis

Repeated measures one-way ANOVA, with a Dunnett's post-test, or a paired *t*-test was used to compare differences between group means as appropriate. A one-tailed Wilcoxon matched pairs test was used to compare differences between group medians. Statistical analyses were performed using GraphPad Prism v4.0 (USA).

3. Results

3.1. Cytotoxic effect of proteins on adenoidal mononuclear cells

Individual and fused proteins were titrated and added to AMNC and viability was assessed after 7 days using flow cytometry. All proliferative responses were measured on day 7, as we have shown

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