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Investigation of nontypeable *Haemophilus influenzae* outer membrane protein P6 as a new carrier for lipooligosaccharide conjugate vaccines

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Abstract

Nontypeable *Haemophilus influenzae* (NTHi) outer membrane protein P6 was used as a new protein carrier for NTHi detoxified lipooligosaccharide (dLOS) conjugates due to its conservation and potential to elicit bactericidal antibodies. P6 was covalently conjugated to dLOS of strain 9274 through adipic acid dihydrazide with different ratios of dLOS to P6, which resulted in two conjugate formulations with weight ratios of dLOS to P6 of 3.7 for dLOS–P6 (I) and 1.6 for dLOS–P6 (II). Binding activity of the conjugates was examined by an enzyme-linked immunosorbent assay with mouse monoclonal antibodies specific to LOS and P6 and a rabbit anti-P6 serum. The results showed that the conjugates bound not only to the LOS antibody but also to both P6 antibodies, suggesting that the conjugates retained epitopes of both LOS and P6 antigens. Animal studies revealed that dLOS–P6 (II) induced high levels of anti-LOS and anti-P6 IgGs in mice and rabbits. However, dLOS–P6 (I) induced lower levels of anti-LOS IgGs in mice and rabbits and anti-P6 IgGs in rabbits with no anti-P6 IgGs in mice. In addition, all rabbit, but not mouse, antisera elicited by the conjugates showed bactericidal activity against the homologous strain, and two of them elicited by each conjugate plus Ribi adjuvant showed cross-bactericidal activity against three of five major serotype stains. These data indicate that P6 could serve as an effective carrier for dLOS or other carbohydrate conjugates and that the ratio of carbohydrate to P6 might contribute to immune responses in vivo.
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Keywords: P6; Carrier; LOS; Conjugate vaccine; Nontypeable *Haemophilus influenzae*

1. Introduction

Nontypeable *Haemophilus influenzae* (NTHi) is one of the leading causative agents of bacterial otitis media in children and a common cause of pulmonary infection in adults with decreased resistance [1]. The success of this organism as a colonizer and pathogen is due to its lack of reliance on any single mechanism of attachment and its ability to respond rapidly to host defense mechanisms by antigenic variation of proteins and enzymes as well as phase variations [2]. To date there is no licensed vaccine against NTHi. Vaccine development is still in the stage of protective antigen identification. An ideal vaccine candidate needs to be exposed to the surface to contact the host immune system and to be the target for specific antibodies and perhaps cytotoxic T-cells. Therefore, primary studies have been focused on outer membrane components of NTHi, which have been shown to have the potential to stimulate protective humoral and cellular immunity against the organism [2,3].

Lipooligosaccharide (LOS) is a major antigenic component on the NTHi cell surface, a virulence factor, as well as a potentially protective antigen [4]. Our previous study showed that coupling detoxified LOS (dLOS) to a carrier protein, tetanus toxoid (TT), elicited high levels of anti-LOS antibodies in mice and rabbits [5]. TT is not only a vaccine but also

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a common carrier for carbohydrate conjugate vaccines due to its safety, stability, and excellent immunogenicity. Thus, most children may have high levels of preexisting antibodies to it after routine immunization programs [6]. Suppression of the immune response could occur with conjugate vaccines using TT as a carrier when anti-TT antibodies preexist from vaccination or with passive maternal antibodies that circulate through the fetus [7,8a]. However, there is controversy on this point [8b]. Previously, total outer membrane proteins (OMPs) and high molecular weight proteins (HMPs) from NTHi were used for the generation of dLOS–protein conjugates. The OMPs were effective carriers in eliciting higher levels of anti-LOS antibodies than a dLOS–TT conjugate, but their quality control was difficult due to variable components of the OMPs [9]. The HMPs were also effective carriers in eliciting anti-LOS antibodies, but the synergetic effect of the HMPs was not significant [10]. In this study, we aimed to extend our experiments to new OMPs derived from NTHi as carriers for dLOS conjugates to circumvent the potential carrier-induced epitope specific suppression and to identify new usable carriers for conjugate vaccines.

P6, an approximate 16-kDa lipoprotein, is a conserved OMP of NTHi and *H. influenzae* (Hi) and comprises 1–5% of the total OMPs [11]. P6 showed a high degree of molecular conservation among strains at both the protein and DNA levels [3,12]. The sequences of P6 from three geographically unrelated strains were 100% homologous at the amino acid level [11]. Portions of P6 were exposed on the bacterial cell surface, and these portions induced an immune response in vivo [13,14]. Furthermore, the constitutive expression of P6 under strong selective pressure on Hi and the inability to construct a Hi P6 mutant suggest that P6 may be important for the organism's survival [14].

P6 has been extensively investigated over the last decade. Antisera to the P6 or recombinant P6 (rP6) were bactericidal to Hi strains and protective in an infant rat model of bacteremia [15]. However, the immunogenicity or protection of rP6 sometimes varied due to possible conformational changes in the protein [3,16], while the purified P6 did induce bactericidal antibodies with protection in a chinchilla model of otitis media [17]. Recent studies showed that intranasal immunization of mice with the P6 plus cholera toxin (CT) resulted in specific immune responses in the middle ear mucosa [18] and enhanced clearance of NTHi from middle ears challenged with NTHi [19]. Intranasal immunization of mice with rP6 plus CT also induced mucosal immune responses and enhanced clearance of NTHi from the mouse nasopharynx [20]. P6 could be a target of human bactericidal antibodies [14]. The levels of maternally acquired human serum antibodies to P6 declined and remained low until two years of age [21], a period that coincided with the peak incidence of otitis media [22]. In addition, P6 may contribute to the activation of nuclear factor (NF)-κB, a transcription factor involving inflammation during NTHi infection [23]. These data suggest that P6 antibodies may provide protection from NTHi infection.

In this study, P6 was conjugated to dLOS for investigation of its feasibility as a new carrier protein for LOS conjugates and to study the synergistic effects of immune responses elicited by both LOS and the P6.

2. Materials and methods

2.1. Bacterial growth and LOS purification

A clinical isolate of NTHi strain 9274 and five major LOS prototype strains were kindly provided by Apicella [24a,b]. Conditions for the bacterial growth were previously described [5]. LOS was purified from the bacteria by a modified phenol–water extraction method [25].

2.2. Detoxification and derivatization of LOS

A hydrazinolysis was used to produce the detoxified LOS (dLOS) [26], then an adipic acid dihydrazide (ADH, Aldrich Chemical Co., Milwaukee, WI) was bound covalently to the dLOS to form an adipic hydrazide derivative, AH-dLOS [27].

2.3. Purification and characterization of P6

P6 was isolated from strain 9274 as described by DeMaria et al. [17]. Protein concentration was determined using a Micro BCA protein kit (Pierce, Rockford, IL). P6 purity was analyzed by SDS–PAGE and Coomassie brilliant staining. The amount of residual LOS in the P6 preparation was estimated by silver staining after SDS–PAGE, using purified LOS as a standard [28a]. Five micrograms of P6 digested by proteinase K was used for SDS–PAGE and silver staining analysis. To further analyze the P6 preparation, a mouse monoclonal antibody (mAb), 4G4, generated from tissue culture supernatant, specific to P6 [28b] was used in enzyme-linked immunosorbent assay (ELISA) since the Western blot with 4G4 was not sensitive enough to detect the P6. In the ELISA, P6, LOS, or bovine serum albumin (BSA) in duplicate were coated overnight on a 96-well Nunc-Immuno^{TM} plate (MaxiSorp^{TM} surface, Nalge Nunc International, Denmark) at a concentration of 10 μg/ml in PBS (pH 7.4) with 10 mM magnesium chloride [5]. The plate was blocked with 1% BSA for 1 h, incubated with 4G4 for 3 h, and then with goat anti-mouse IgG-alkaline phosphatase conjugate for 2 h, followed by the addition of a substrate, *p*-nitrophenyl disodium phosphate. Optical density was obtained as ELISA readings at A_{405} after 90 min of incubation. All steps were performed at room temperature with four washings between steps.

2.4. Preparation of dLOS–P6 conjugates

To minimize the intermolecular cross-linking of P6 in the conjugation reaction, the amine groups of P6 (1 mg/ml)