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Conjugation of PEG-hexadecane markedly increases the immunogenicity of pneumococcal polysaccharide conjugate vaccine

Xin Chang [a,b,1], Weili Yu [b,1], Shaoyang Ji [b], Lijuan Shen [b], Aijuan Tan [a,*], Tao Hu [b,*]

[a] College of Pharmacy, University of Guizhou, Guiyang 550025, Guizhou Province, China
[b] State Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, China

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ABSTRACT

Streptococcus pneumoniae is a serious Gram-positive pathogen that can lead to an invasive pneumococcal disease with high mortality rate. Pneumococcal capsular polysaccharide (PS) is a key virulence determinant and its immunogenicity can be increased by conjugation with a carrier protein. However, the PS-specific cellular and humoral immunity of pneumococcal conjugate vaccine needs further improvement. Hexadecane (HD) is an element of lipid that decorates the surface of nearly all microbial classes. Polyethylene glycol (PEG)-HD conjugate (PEG-HD) is soluble and can act as an adjuvant. In the present study, a novel pneumococcal polysaccharide conjugate vaccine was prepared by conjugation of tetanus toxoid (TT) portion of PS-TT conjugate (PS-TT) with PEG-HD. As compared with PS-TT, conjugation with PEG-HD led to an 8.0-fold increase in the PS-specific IgG titers. Conjugation with PEG-HD also gave rise to 34.9-, 3.6- and 7.7-fold increase in the IFN-γ, TNF-α and IL-5 levels, respectively. Thus, the conjugated PEG-HD has a stimulatory adjuvant activity to potentiate a robust humoral and cellular immunity. Our proposed conjugate was expected to act as an effective pneumococcal conjugate vaccine for prevention of *S. pneumoniae* infections.

1. Introduction

Streptococcus pneumoniae is a serious Gram-positive pathogen that can give rise to invasive pneumococcal diseases (IPDs) with high mortality rate [1]. Due to the increased resistance of pneumococcal strains to antimicrobial agents, effective vaccination is urgently needed to prevent *S. pneumoniae* infections [2]. Pneumococcal capsular polysaccharide (PS) is a key virulence determinant that has been used as a preventive vaccine [3]. Pneumococcal conjugate vaccine has been developed by conjugation of pneumococcal capsular PS with a carrier protein [4]. For example, a 13-valent pneumococcal conjugate vaccine (PCV-13, Pfizer Inc., USA) efficiently reduces the incidence of IPDs and colonization via the acquisition of PS-specific antibodies [5]. Attempts to improve the level of PS-specific antibodies have been conducted by optimization of PS-to-protein ratio, carrier protein, conjugation chemistry, and spacer arm between PS and protein [6–9]. For instance, a meningococcal conjugate vaccine with a spacer arm of polyethylene glycol (PEG) could elicit higher PS-specific IgG titers than the one without PEG [8].

Recent studies suggest that acquired immunity to *S. pneumoniae* is derived not only from natural acquisition of capsular PS-specific antibodies against IPDs, but also at least from the pneumococcus-specific $CD4^+$ TH17 cells that reduces the duration of carriage [10]. The important role of cellular immunity in host and *S. pneumoniae* interactions has been pointed out, such as shaping the type of inflammation ensuing from infection [11]. In order to provide full protection against *S. pneumoniae*, a novel pneumococcal conjugate vaccine was urgently needed to potentiate a potent PS-specific humoral and cellular immunity. However, the cellular immunity has been neglected for development of pneumococcal conjugate vaccines.

Adjuvants have been used to augment a particular immune response to antigens [12]. Adjuvants with proper delivery approach are needed for development of conjugate vaccines [13]. Interestingly, conjugation of conjugate vaccine and adjuvant in one delivery system was considered to be more efficient than co-administration of them to improve the stimulatory adjuvant activity [14,15]. Recently, β-glucan and Toll like receptor 7 agonists (TLR7a) were used for conjugation of meningococcal conjugate vaccines and markedly improved the PS-specific immunogenicity [14,15].

* Corresponding authors at: National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, No. 1 Bei-Er-Tiao Street, Haidian District, Beijing 100190, China.
E-mail address: thu@ipe.ac.cn (T. Hu).
[1] The two authors contribute equally to this work.

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Lipids decorate the surface of nearly all microbial classes and can act as adjuvants. For example, monophosphoryl lipid A (MPL) promotes primarily a Th1 response characterized by increased IFN-γ production and induction of IgG2a in murine [16]. Cell wall mycolic acid (C_{60}-C_{90} fatty acid) from *Mycobacterium tuberculosis* is lipid antigen with stimulatory adjuvant activity [17]. As insoluble adjuvants, these lipids are typically mixed with antigens and not suitable for conjugation of antigens. Hexadecane (C_{16}, HD) is structurally similar to MPL and mycolic acid, and becomes water-soluble by conjugation with PEG. The PEG-HD conjugate (PEG-HD) has been used for conjugation of human growth hormone (hGH), which improved the pharmacological property of hGH [18].

In the present study, pneumococcal capsular PS serogroup 18C was conjugated with tetanus toxoid (TT) to obtain a conjugate (PS-TT). A novel conjugate vaccine (PS-TT-P-HD) was prepared by conjugation of TT portion of PS-TT with PEG-HD. As compared with PS-TT, PS-TT-P-HD was demonstrated to potentiate more robust PS-specific humoral and cellular immunity. Thus, conjugation with PEG-HD could markedly improve the PS-specific immunogenicity of PS-TT.

2. Materials and methods

2.1. Materials

Hexadecylamine, *N*-(2-aminoethyl)maleimide (AM), and 2-iminothiolane (IT) were purchased from Sigma (USA). Horse radish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (HRP-IgG), IgG1 antibody (HRP-IgG1), IgG2a antibody (HRP-IgG2a), and IgM antibody (HRP-IgM) were ordered from Abcam (USA). RPMI 1640 medium and fetal bovine serum (FBS) were obtained from Gibco (USA). Maleimide PEG *N*-hydroxysuccinimide ester with Mw of 3.5 kDa (mal-PEG-NHS) and methoxyl PEG maleimide with Mw of 3.5 kDa (mal-PEG) were ordered from Jenkem Biotech (China). IFN-γ, TNF-α, and IL-5 ELISA kits were purchased from eBioscience (USA). Pneumococcal PS serogroup 18C and TT were kindly provided by Hualan Biological Engineering Inc. (China).

2.2. Preparation of PS-TT and PS-TT-P

PS (4 mg/ml, 6 ml) was oxidized by 10 mM sodium periodate in 20 mM acetate buffer (pH 5.8) for 40 min at room temperature (Fig. 1). The oxidized PS (3 mg/ml, 6 ml) was incubated with AM (6 mg/ml, 3 ml) and $NaCNBH_3$ (9 mg/ml, 1 ml) in 20 mM phosphate buffer (pH 7.4, PB) at 4 °C overnight, followed by extensive dialysis against PB. TT (3 mg/ml, 6 ml) was incubated with 100-fold molar excess of IT in PB at 4 °C overnight, followed by incubation with the activated PS (2 mg/ml, 9 ml) (Fig. 1). The PS-TT conjugate (PS-TT, 3 mg PS/ml, 2 ml) was incubated with mal-PEG (3 mg) in PB at 4 °C overnight to obtain the PS-TT-PEG conjugate (PS-TT-P) (Fig. 1).

2.3. Preparation of PS-TT-P-HD

Hexadecylamine (1 mg) in *N*,*N*-dimethyl formamide was incubated with 3 mg mal-PEG-NHS at room temperature for 5 h. The reaction mixture (2 ml) was extensively dialyzed with PB, followed by addition of PS-TT (3 mg PS/ml, 2 ml) at 4 °C overnight to obtain the conjugate (PS-TT-P-HD) (Fig. 1).

2.4. Purification of the conjugates

PS-TT, PS-TT-P and PS-TT-P-HD were purified by a Superdex 200 column (1.6 cm × 60 cm, GE Healthcare, USA), based on size exclusion chromatography. The column was equilibrated and eluted with PB at a flow rate of 2.0 ml/min. The peaks corresponding to the three conjugates were fractionated and concentrated.

2.5. Quantitative assay

PS contents of the conjugates were assayed by phenol sulfuric acid. TT contents of the conjugates were measured by

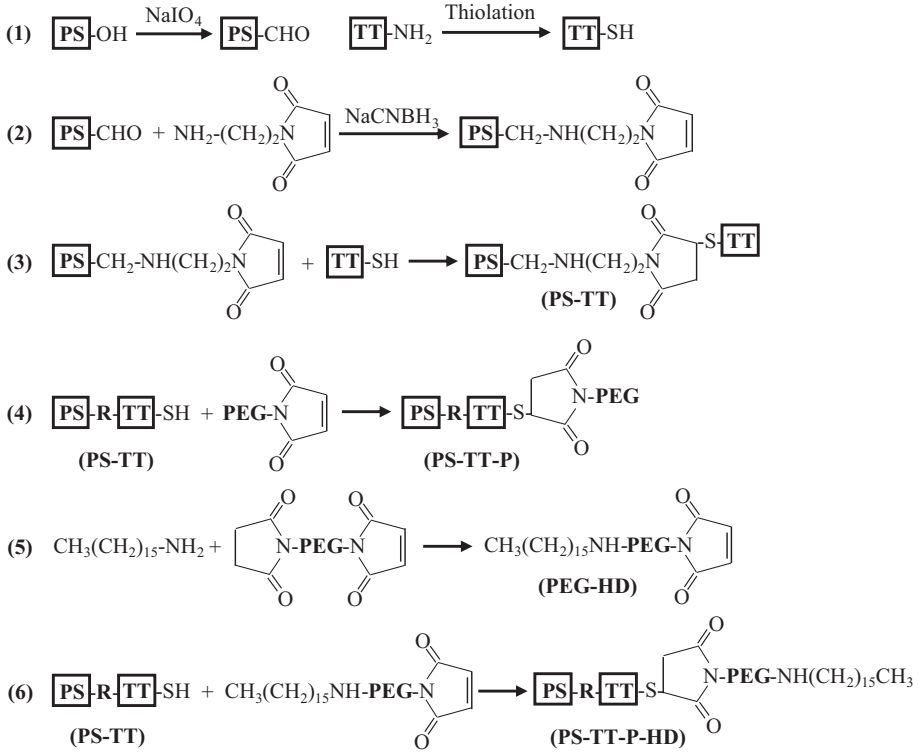

Fig. 1. Schematic representation of PS-TT, PS-TT-P and PS-TT-P-HD.

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