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Nasal vaccination with pneumococcal surface protein A in combination with cationic liposomes consisting of DOTAP and DC-chol confers antigen-mediated protective immunity against *Streptococcus pneumoniae* infections in mice

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ABSTRACT

Infectious diseases are the second leading cause of death worldwide, suggesting that there is still a need for the development of new and improved strategies for combating pathogens effectively. *Streptococcus pneumoniae* is the most virulent bacteria causing pneumonia with high mortality, especially in children and the elderly. Because of the emergence of antibiotic resistance in *S. pneumoniae*, employing a serotype-independent mucosal vaccine would be the best approach to prevent and treat the diseases caused by *S. pneumoniae*. In this study, we have developed a pneumococcal nasal vaccine, consisting of pneumococcal surface protein A (PspA) and cationic liposomes composed of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and cholesteryl 3β-*N*-(dimethylaminoethyl)carbamate (DC-chol) (DOTAP/DC-chol liposome). The efficiency of this cationic liposome-based PspA nasal vaccine was examined in a murine model of *S. pneumoniae* infection. Intranasal vaccination with PspA and DOTAP/DC-chol liposomes conferred protective immunity against lethal inhalation of *S. pneumoniae*, improving the survival rate of infected mice. Moreover, intranasal immunization with PspA and DOTAP/DC-chol liposomes not only induced the production of PspA-specific IgA and IgG by both mucosal and systemic compartments but also elicited PspA-specific Th17 responses, which play a pivotal role in controlling *S. pneumoniae* infection by host innate immune response. We further demonstrated that DOTAP/DC-chol liposomes enhanced PspA uptake by nasal dendritic cells (DCs), which might be a mechanism for the induction of protective immune responses to *S. pneumoniae* infection. These results show that DOTAP/DC-chol liposome would be an efficient mucosal vaccine system for a serotype-independent universal nasal vaccine against pneumococcal infection.

1. Introduction

Infectious diseases are the second leading cause of death worldwide, suggesting that the development of new agents and/or strategies for combating the pathogens is still a challenge [1]. The emergence of numerous antibiotic resistant microbes renders the usage of vaccines as the fundamental mechanism for treatment and prevention of these communicable diseases [2].

Streptococcus pneumoniae is a major virulent pathogenic bacterium causing pneumonia, meningitis, vaginitis and septicemia responsible for a high mortality rate, especially in children and the elderly. The emergence of antibiotic-resistant *S. pneumoniae* strains rendered the disease management even more a challenging prospect [3]. Recent estimates indicate that the diseases caused by *S. pneumoniae* are responsible for 3–5 million deaths per year [4], despite the current availability of vaccines, such as 23-valent pneumococcal polysaccharide vaccine (PPSV-23) and 13-valent pneumococcal conjugate vaccine (PCV-13), against *S. pneumoniae* infection [5]. The problems

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pertaining to the existing pneumococcal vaccines in clinical usage are following: i) the protective effects induced by these pneumococcal polysaccharide vaccines are serotype specific, as the capsular polysaccharide, expressed in the outer most layer of *S. pneumoniae*, is structurally distinct among the different strain serotypes [6, 7], ii) the pneumococcal vaccine based on the capsular polysaccharides is ineffective against the infections of serologically non-typeable *S. pneumoniae* (also termed as non-encapsulated *S. pneumoniae* (NESp) due to the lack of a capsule), which are reported to be on rise since the launch of polysaccharide vaccines [8], iii) the children and the elderly, the major target group for pneumococcal vaccines, inherently respond poorly to polysaccharide antigens due to the lack of T cells memory [9], iv) polysaccharide antigens inadequately elicit long-lasting immune memory responses [10], and v) the currently available pneumococcal vaccines are administered as either intramuscular or subcutaneous injection. Although these immunization routes elicit a strong systemic immune response, it fails to elicit a mucosal immune response especially in the nasopharyngeal region, which is an important region for entry and colonization of *S. pneumoniae* [11]. To overcome the issues stated above, it is necessary to develop a universal pneumococcal mucosal vaccine based on antigenic proteins present in all *S. pneumoniae* strains, which can confer antigen-mediated protective immunity against *S. pneumoniae* infection in a serotype-independent fashion.

Pneumococcal surface protein A (PspA) is expressed in all pneumococcal strains isolated so far and is highly variable in terms of their nucleotide sequences among *S. pneumoniae* strains. PspA families are classified into three families and six clades (family 1, clades 1 and 2; family 2, clades 3–5; family 3, clade 6). At least 98% of clinical isolates belong to families 1 and 2 [12]. Despite the variability in nucleotide sequences of PspA, it has been established that PspA-specific immune responses conferred serotype-independent protective immunity against *S. pneumoniae* infection [13]. In particular, PspA recombinant protein derived from Rx1 (family 1, clade 2) induces significant cross-reactivity to PspA families 1 and 2 [14, 15]. Hence, PspA would be a promising candidate for vaccine development [16]. The use of a mucosal vaccine system would be ideal as controlling the colonization of the bacteria in the nasal cavity is the pivotal requirement for prevention and treatment of *S. pneumoniae* infection. Although mucosal vaccines are encouraged for treatment and prevention of infectious diseases caused by bacteria, fungi, and viruses, very few mucosal vaccines are currently available for clinical use, partly due to the lack of safe and effective mucosal adjuvants [17–19]. Since protein antigens show inherently poor immunogenicity when administrated via mucosal route [20], inclusion of a suitable mucosal adjuvant in the formulation is critical for induction of immune responses to the antigen effectively.

We have recently elucidated that intranasal administration of an antigenic protein with the cationic liposomes, composed of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and cholesteryl 3β-*N*-(dimethylaminoethyl)carbamate (DC-chol) (termed as DOTAP/DC-chol liposomes), resulted in both systemic and mucosal antigen-specific antibody responses in mice, at least partly, because of the enhancement in the delivery of antigenic proteins to antigen-presenting cells (APCs) such as dendritic cells (DCs) in the nasal region. This indicated that DOTAP/DC-chol liposomes would act as a mucosal adjuvant for nasal vaccines against infectious diseases [21, 22]. In addition, we have also revealed that DOTAP/DC-chol liposomes can induce the production of antigen-specific Th2 and Th17 cytokines from the cells isolated from vaccinated mice, indicating that DOTAP/DC-chol liposome can serve as a mucosal adjuvant to promote Th2/Th17 response [unpublished results]. We have hypothesized that the DOTAP/DC-chol liposomes might be appropriate as a mucosal adjuvant for nasal pneumococcal vaccines because of the following features: i) intranasal administration of DOTAP/DC-chol liposomes enhances the antigen specific mucosal IgA response, which in turn would lead to inhibitory effects on *S. pneumoniae* colonization upon infection, ii) intranasal administration of DOTAP/DC-chol liposomes induces Th17 responses [unpublished results], and it is well documented that antigen-specific Th17 immunity is important for the elimination of *S. pneumoniae* [23], and iii) intranasal immunization of DOTAP/DC-chol liposomes did not show any toxic effects in mice *in vivo* [21].

In the present study, we have examined the protective effects of a cationic liposome-based nasal pneumococcal vaccine using PspA as an antigen against *S. pneumoniae* infection. Additionally, we have also investigated whether these cationic liposomes promote PspA uptake by DCs in the nasopharyngeal compartment, in order to understand the potential mechanism(s) of the mucosal adjuvant effects exerted by DOTAP/DC-chol liposomes.

2. Materials and methods

2.1. Animal handling

Female BALB/c mice (8 weeks old) were obtained from Japan SLC (Shizuoka, Japan). The mice were housed in a specific pathogen-free (SPF) environment. All animal experiments were conducted following the guidelines for laboratory animal experimentations of Tokyo University of Pharmacy and Life Sciences and the guidelines of Animal Care and Use Committee of National Institutes of Biomedical Innovation, Health and Nutrition. Each experimental protocol was approved by a committee for laboratory animal experimentations at these institutions (P14–31, P15–80, P16–09, P17–26, and #DS25–3R8). The bacterial infections were performed under anesthesia with isoflurane gas and all the efforts were taken to minimize the suffering of mice.

2.2. Reagents

The cationic lipids, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and cholesteryl 3β-*N*-(dimethylaminoethyl)-carbamate (DC-chol) were purchased from Avanti Polar Lipids (Alabama, USA).

2.3. Preparation of liposomes

The liposomes were prepared as described earlier [21]. Briefly, 10 μmol of total lipids (DOTAP:DC-chol at 1:1 mol ratios) were evaporated to dryness in a glass tube and desiccated for at least 1 h *in vacuo*. The lipid films obtained were hydrated by the addition of 250 μl of phosphate-buffered saline (PBS) and then vortexed for 5 min at 25 °C. The prepared multilamellar vesicles were extruded 10 times by passing through a 100-nm-pore polycarbonate membrane (Advantec, Tokyo, Japan) and then sterilized by filtration through 0.45-μm filter membranes (Iwaki, Tokyo, Japan). The particle size and ζ-potential of the liposomes were measured by Nicomp 380 ZLS (Particle Sizing Systems, Port Richey, USA). The liposomes used in this study had a particle size of 137.9 ± 11.6 nm with a ζ-potential of 4.0 ± 2.1 mV.

2.4. Recombinant PspA expression and purification

PspA fragment was amplified by polymerase chain reaction (PCR) (forward primer: 5′-ATGATGATGCATATGGAAGAATCTCCCGTA GCC-3′, *Nde*I site is underlined; reverse primer: 5′-GCGGGATCCTTAT TCTGGGGCTGGAGTTTC-3′ *Bam*HI site is underlined) from pET20b-PspA template containing PspA from *S. pneumoniae* Rx1 (PspA family 1 and clade 2) [24]. The PspA PCR fragment was cloned into pET16b expression vector (Novagen, Darmstadt, Germany), after digesting both the vector and the insert with *Nde*I and *Bam*HI, for the production of recombinant PspA protein (PspAp). The prepared pET16b-PspA plasmid was transformed into *Escherichia coli* strain BL21 (DE3). The transformed cells were grown in LB medium containing ampicillin at 37 °C and the recombinant protein production was induced with 0.25 mM isopropyl-D-thiogalactopyranoside (IPTG; Nacalai Tesque, Kyoto, japan) for 3 h at 37 °C. The harvested cells were re-suspended in buffer A containing 10 mM Tris-HCl (pH 8.0), 400 mM NaCl, 5 mM $MgCl_2$,