Chemically Synthesized *Alcaligenes* Lipid A Shows a Potent and Safe Nasal Vaccine Adjuvant Activity for the Induction of *Streptococcus pneumoniae*-Specific IgA and Th17 Mediated Protective Immunity

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Abstract

Effective and safe vaccine adjuvants are needed to appropriately augment mucosal vaccine effects. Our previous study demonstrated that lipopolysaccharide (LPS) from Peyer's patch resident *Alcaligenes* stimulated dendritic cells to promote the production of mucosal immunity-enhancing cytokines (e.g., IL-6 and BAFF), thus enhancing antigen-specific immune responses (including IgA production and Th17 responses) without excessive inflammation. Here, we chemically synthesized *Alcaligenes* lipid A, the biologically active part of LPS, and examined its efficacy as a nasal vaccine adjuvant for the induction of protectively immunity against *Streptococcus pneumoniae* infection. Mice were nasally immunized with pneumococcal surface protein A (PspA) as a vaccine antigen for *S. pneumoniae*, together with *Alcaligenes* lipid A. *Alcaligenes* lipid A supported the generation of high levels of PspA-specific IgA and IgG responses through the augmentation of germinal center formation in the nasopharynx-associated lymphoid tissue and cervical lymph nodes (CLNs). Moreover, *Alcaligenes* lipid A promoted PspA-specific $CD4^+$ Th17 responses in the CLNs and spleen. Furthermore, neutrophils were recruited to infection sites upon nasal infection and synchronized with the antigen-specific T and B cell responses, resulting in the protection against *S. pneumoniae* infection. Taken together, *Alcaligenes* lipid A could be applied to the prospective adjuvant to enhance nasal vaccine efficacy by means of augmenting both the innate and acquired arms of mucosal immunity against respiratory bacterial infection.

Keywords: ***Alcaligenes* lipid A**; **IgA antibody**; **neutrophil**; **pneumococcal surface protein A (PspA), *Streptococcus pneumoniae***; **Th17 response**

Introduction

Various pathogens cause infectious diseases by invading through the surface of the respiratory and gastrointestinal tracts [1]. It is therefore important to induce protective immunity at mucosal sites to prevent these diseases. In vaccine development, the injection-type vaccines can induce systemic immune responses but not mucosal immune responses, whereas mucosal vaccines (e.g., nasal and oral vaccines) can induce both systemic and mucosal immune responses [2]. Therefore, mucosal vaccines can be considered to protect from infectious diseases caused by mucosally invading pathogens.

In the respiratory immune system, nasopharynx-associated lymphoid tissue (NALT) has been suggested to be one of target tissues for the delivery of nasal vaccine, since it is equipped with all of the necessary immune cells for the induction of antigen-specific immune responses. In murine nose, NALT is found as on both sides of the nasopharyngeal duct, dorsal to the cartilaginous soft palate, and it is considered analogous to Waldeyer's tonsillar ring in humans [3].

Materials and Methods

2.1. Mice

Female Balb/c mice (age, 6 or 7 weeks) were purchased from CLEA Japan (Tokyo, Japan) and kept for at least 1 week before the experiments. All experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of the National Institutes of Biomedical Innovation, Health and Nutrition (NIBIOHN) and the Committee on the Ethics of Animal Experiments of NIBIOHN (approval no. DS25-2, DS25-3). In accordance with the guidelines, murine condition was checked at least once a day and the mice were euthanized if their body weight reduction exceeded 20%.

2.2. Preparation of PspA Protein

The PspA gene was amplified by polymerase chain reaction and cloned into pET16b plasmid Darmstadt, as previously described, to yield pET16b-PspA plasmid [17]. To obtain PspA recombinant proteins, the plasmids were transformed into *E. coli* strain BL21 . Protein production was induced by adding isopropyl-β-D-thiogalactopyranoside (Nacalai Tesque, Kyoto, Japan). The culture pellets were sonicated for 1 min three times in buffer A (10 mM Tris-HCl [pH 8.0], 400 mM NaCl, 5 mM $MgCl_2$, 0.1 mM PMSF, 1 mM 2-mercaptethanol, and 10% glycerol). After centrifugation of the mixture at 4 °C and 17,800× *g* for 15 min, the supernatants were filtered through a 0.45 μm Millex-HV filter unit and loaded into HiTrap HP columns Healthcare Pittsburgh was eluted with buffer A containing 100 to 500 mM imidazole.

Results

3.1. Alcaligenes Lipid A Enhances Nasally-Induced PspA-Specific Mucosal Immune Responses through the Formation of Germinal Centers in the NALT

We first examined whether *Alcaligenes* lipid A promoted antigen-specific immune responses in the respiratory tracts of female Balb/c mice aged about 8 weeks. After nasal immunization with PspA together with *Alcaligenes* lipid A,

nasal wash and bronchoalveolar lavage fluid were collected to evaluate PspA-specific antibodies by enzyme-linked immunosorbent assay . Mice nasally-immunized with PspA and *Alcaligenes* lipid A showed higher levels of PspA-specific IgA antibodies in the nasal wash and BALF than mice immunized with PspA alone (**Figure 1**A,B), demonstrating that co-administration of *Alcaligenes* lipid A supported the generation of elevated antigen specific-immune responses both in upper and lower respiratory tracts. The IgA enhancing adjuvant effects of *Alcaligenes* lipid A were a dose-dependent manner (**Figure S1**). Notably, *Alcaligenes* lipid A showed stronger IgA enhancing adjuvant activity than the classically used mucosal adjuvant cholera toxin [19].

Discussion

New mucosal adjuvants are needed to augment the immune responses when it is mucosally vaccinated with low-antigenic-subunit-ones. Moreover, it is rising the necessity of developing mucosal vaccines to protect various infectious diseases. We previously found that *Alcaligenes* LPS acts as a weak TLR4 agonist and enhances antigen-specific immune responses without excessive inflammation[10]. In this study, we extended these findings by showing that chemically synthesized *Alcaligenes* lipid A has strong potential as a safe and an effective nasal vaccine adjuvant to augment PspA-specific immune responses, and to prevent respiratory pneumococcal infection. Recently, a lipid A-based vaccine adjuvant has been developed. For example, 3-O-desacyl-4′-monophosphoryl lipid A (MPLA), a derivative of lipid A derived from *Salmonella minnesota* R595 LPS, is used for enhancement of the efficacy of Hepatitis B virus; human papillomavirus vaccine [26,27,28]. *S. minnesota* is a pathogenic bacteria, so its lipid A needs to be chemically modified to reduce its pathogenicity by deficient of phosphoryl group. In contrast, *Alcaligenes* is lymphoid resident commensal bacteria, thus, its lipid A could be applied to a vaccine adjuvant without chemical modification, and enhance antigen-specific immune responses without excessive inflammation.

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